Gait Alterations and Synaptic lncRNA Expression Aberrations in a Genetic Rat Model of ASD: Neurexin1 Mutant

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Submitted by:

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UNDER THE SUPERVISION OF

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CERTIFICATE

This is to certify that the dissertation report entitled **'Gait Alterations and Synaptic lncRNA Expression Aberrations in a Genetic Rat Model of ASD: Neurexin1 Mutant'** is the result of the work carried out by **Ms. Gauri Shintre** in the Division of Cellular and Molecular Neuroscience, National Brain Research Centre, Manesar, Haryana, India, for partial fulfilment of requirement for the M.Sc. degree.

The work presented herein is original and has not been submitted previously for the award of any degree or diploma to the National Brain Research Centre (Deemed to be University) or any other University. This work is based on the guidelines of the National Brain Research Centre (Deemed to be University) and is a record of the candidate's own efforts.

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DECLARATION BY CANDIDATE

I, Gauri Shintre, hereby declare that the work presented in the dissertation report entitled, 'Gait Alterations and Synaptic lncRNA Expression Aberrations in a Genetic Rat Model of ASD: Neurexin1 Mutant'

Was carried out by me under the guidance of Dr. Sourav Banerjee, Division of Cellular and Molecular Neuroscience, National Brain Research Centre (Deemed to be University), Manesar, Haryana, India.

I declare that no part of the thesis contains plagiarized material, and any previously published or other material sourced from anywhere else has been appropriately attributed to the source.

I also declare that no part of this thesis has been previously submitted for award of any degree or diploma to the National Brain Research Centre (Deemed to be University) or to any other University.

GAURI SHINTRE

Place: Manesar

Date: 23.06.25

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List of Abbreviations:

ASD: Autism Spectrum Disorder

PF: Parallel Fibre

PC: Purkinje Cell

CF: Climbing Fibre

DSM: Diagnostic and Statistical Manual of Mental Disorders

PDD: Pervasive Mental Disorders

ICD: International Classification of Diseases

CDD: Childhood Disintegrative Disorder

PDD-NOS: Pervasive Developmental Disorder-Not Otherwise Specified

GWAS: Genome-Wide Association Studies

PCR: Polymerase Chain Reaction

qPCR: Quantitative Polymerase Chain Reaction

DMSO: Dimethyl Sulfoxide

NFW: Nuclease Free Water

dNTP: Deoxynucleotide Triphosphate

ZFN: Zinc Finger Nuclease

KO: Knock Out

Nrxn: Neurexin gene

TBS: Tris Based Saline

SN: Synaptoneurosome

TL: Total Lysate

DCN: Deep Cerebellar Nuclei

IO: Inferior Olive

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Abstract

Neurexin1 is a presynaptic cell adhesion protein implicated in the etiology of autism spectrum disorder (ASD), with known roles in synaptic function and plasticity. Interestingly, people with ASD are more likely to have heterozygous deletions or loss-of-function mutations in Neurexin1 than homozygous disruptions, underscoring the value of heterozygous models in researching the underlying mechanisms of illness. In this thesis, I investigated both the molecular and behavioural alterations in a heterozygous Neurexin1 knockout (KO) rat model to explore the downstream consequences of Neurexin1 disruption.

Using quantitative PCR, I found a significant reduction in the expression of the lncRNA GAS5, while PVT1 expression levels were not found to be significantly reduced in Neurexin1 heterozygous mutant males. GAS5 is known to act as a molecular sponge for miR-21 and regulates the AKT signaling pathway via PTEN, suggesting that its downregulation may alter cellular growth and synaptic plasticity pathway. GAS5 also interacts with synapse-associated proteins such as CAPRIN1 and G3BP2, implicating it in the regulation of local protein synthesis at the synapse.

To evaluate motor behavior, I assessed spontaneous, unforced gait in a naturalistic setup. Although no statistically robust genotype-dependent differences were observed in stride length or stride duration, a trend toward reduced limb duty factor in heterozygous rats suggested possible alterations in the temporal structure of gait. Considering the complex, nested nature of data, the Linear mixed effects model was applied to identify contributing factors to the gait parameters. Speed significantly influenced gait parameters such as stride length, stride duration, and limb duty factor, aligning with existing biomechanical literature. However, the models accounted for only a modest proportion of variance (marginal $R^2 \sim 0.1$), likely reflecting the limited sample size.

Collectively, the results suggest a potential link between molecular alterations and behavioural outcomes in Neurexin1 heterozygous models, wherein dysregulation of GAS5 may influence synaptic processes and contribute to subtle motor alterations. This integrated molecular and behavioural analysis underscores the relevance of lncRNA regulatory networks, such as those involving GAS5, in the context of ASD. Future studies will aim to increase statistical power and dissect the downstream signaling cascades affected by GAS5.

Chapter 1: Literature Review

1.1. Autism Spectrum Disorder (ASD)

1.1.1. Autism and its history

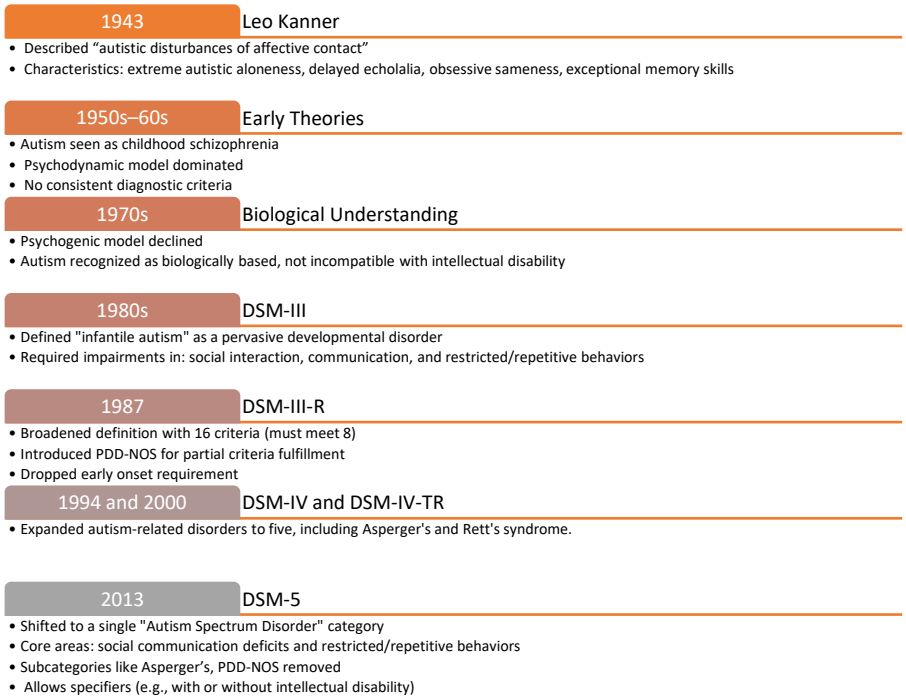
1943
Leo Kanner
• Described “autistic disturbances of affective contact”
• Characteristics: extreme autistic aloneness, delayed echolalia, obsessive sameness, exceptional memory skills
1950s–60s
Early Theories
• Autism seen as childhood schizophrenia
• Psychodynamic model dominated
• No consistent diagnostic criteria
1970s
Biological Understanding
• Psychogenic model declined
• Autism recognized as biologically based, not incompatible with intellectual disability
1980s
DSM-III
• Defined "infantile autism" as a pervasive developmental disorder
• Required impairments in: social interaction, communication, and restricted/repetitive behaviors
1987
DSM-III-R
• Broadened definition with 16 criteria (must meet 8)
• Introduced PDD-NOS for partial criteria fulfillment
• Dropped early onset requirement
1994 and 2000
DSM-IV and DSM-IV-TR
• Expanded autism-related disorders to five, including Asperger's and Rett's syndrome.
2013
DSM-5
• Shifted to a single "Autism Spectrum Disorder" category
• Core areas: social communication deficits and restricted/repetitive behaviors
• Subcategories like Asperger’s, PDD-NOS removed
• Allows specifiers (e.g., with or without intellectual disability)

Figure 1. Historical progression of how autism has been defined and classified in psychiatric diagnostic manuals, reflecting shifting perspectives on core symptoms and diagnostic boundaries.

Leo Kanner initially reported a group of kids with "autistic disturbances of affective contact" in 1943. Since then, our knowledge of autism has changed significantly, from a rare childhood disorder to a spectrum that impacts millions of people worldwide. With roots in behaviour, brain development, and genetics, autism spectrum disorder (ASD) is now understood to be a complex neurodevelopmental disorder that is highly heritable. Autism Spectrum Disorder or ASD is a highly heritable heterogeneous neurodevelopmental disorder characterized by social and communication deficits, alongside repetitive behaviour and restrictive patterns of behaviour and interests (Loukusa, 2021). Individuals with ASD can have varying levels of intellectual ability, with some performing much worse than the general population while others have savant-like capabilities. Unusual gait, atypical posture, clumsiness, coordination issues, and

hyperflexible joints are also exhibited by children with ASD (Fournier et al., 2014; Ganai et al., 2025).

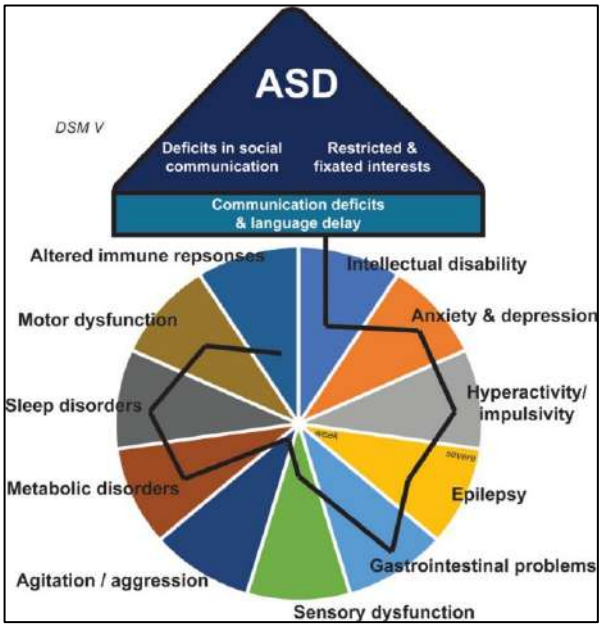

Figure 2. *Clinical features of ASD. Adapted from (Sauer et al., 2021).*

The definition of Autism has undergone severe refinement over the past century, Bleuler in 1911 first characterized autistic thinking as replacing reality with infantile fantasies and hallucinations, as a childhood onset of schizophrenia (Bleuler, 1950). Infantile Autism and Asperger's syndrome were two related disorders distinct from schizophrenia described by Leo Kanner and Hans Asperger. It was in the 1970s when the first genetic tests for autism were done that Michael Rutter claimed that 'autism is a deficiency of fantasy rather than an excess' that brought on a radical change in the understanding of autism (Rutter, 1972). In 1980, the Diagnostic and Statistical Manual of Mental Disorders (DSMIII) completely removed the category of 'Childhood Schizophrenia' and introduced Pervasive Developmental Disorders (PDD) with its four sub-categories which included, 'infantile autism', 'childhood onset pervasive developmental disorder', 'residual autism' and an atypical form (Volkmar et al., 2014). The introduction of 'theory of mind' research was a pivotal moment in autism research. It earmarked a departure from the earlier simple observation of social deficits to understanding the cognitive mechanism of these. Although there are schools of thought that propose alternative perspectives, for instance, Peter Hobson posited that Theory of mind difficulties are secondary to a primary impairment in early emotional engagement. However, even Hobson's work largely refrains from drawing parallels between autism and schizophrenia in terms of hallucinatory or fantasy-based

thinking (*Against the Theory of 'Theory of Mind' - Hobson - 1991 - British Journal of Developmental Psychology - Wiley Online Library*, n.d.; Ganji et al., n.d.). Once a subject of debate, the connection between autism and schizophrenia is now being re-examined by researchers like Judith Rapport and colleagues (Rapoport et al., 2009), who suggest that autism might be a risk factor for schizophrenia.

Being a spectrum disorder, the presentation of symptoms and their severity vary widely among autistic individuals (Lord et al., 2020) a recognition formalised by DSM-5 which was released in 2013. s diagnostic frameworks have evolved to better capture the heterogeneity of autism, a growing body of research and advocacy has begun to reframe these differences not as deficits, but as part of the natural variation in human neurocognitive functioning. The intricacy of the structure and function of the human brain underscores the likelihood that differences in cognitive strengths and weaknesses represent natural human diversity rather than underlying pathology.

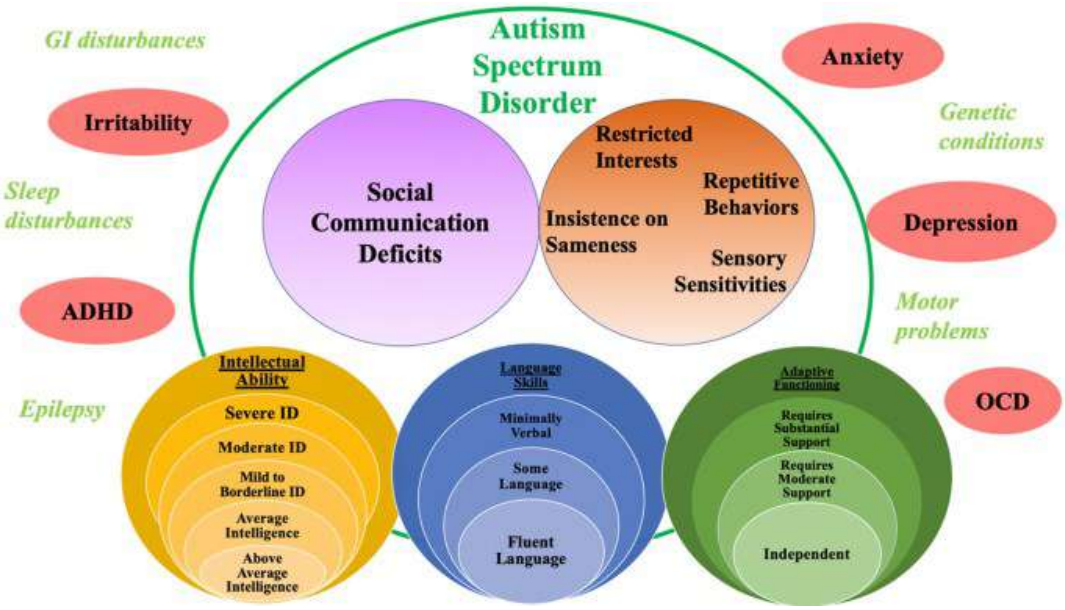

Figure 3.Core Symptoms of Autism Spectrum Disorder adapted from (Lord et al., 2020)

1.1.2. Aetiology

A Sweden-based cohort study estimated 83% of causality due to genetic factors, leaving 17% to environmental factors (Sandin et al., 2017). Initial twin studies conducted in 1977 first suspected genetic inheritance of autism (Folstein & Rutter, 1977). Frazier et al. (2014) demonstrated that extreme autism symptom levels are

strongly heritable, with identical twins showing an 80% concordance rate compared to 40% in fraternal twins (Frazier et al., 2014). Additionally, the risk of a child getting affected was found to be proportional to the percentage of the genome they shared with an affected sibling or parent (Goin-Kochel et al., 2007). These were the seminal studies that established a genetic component to autism, though which genes were involved was still not known.

Early karyotype studies began identifying chromosomal regions potentially involved in ASD, later linkage analysis highlighted loci on chromosomes 7q,1p, 3q, 16p, and 15q. Initially, candidate gene studies focused on neurodevelopmental genes in these regions, such as Hox and Wnt families, but many yielded inconclusive results. From 2001, more consistent associations emerged, implicating genes like RELN, ERX, MECP2, NLGN3, NLGN4, TSC2, and UBE3A in ASD aetiology (Rylaarsdam & Guemez-Gamboa, 2019).

With the advent of sequencing in early 2000, GWAS studies confirmed that the aetiology of ASD was multigenic and highly heterogeneous. Only a small number of ASD are caused by mutations in a single gene. These include Rett syndrome, Fragile X Syndrome, Tuberous Sclerosis, and Schuurs-Hoeijmakers syndrome (Artuso et al., 2011; Stern et al., 2017; Woodbury-Smith & Scherer, 2018). Autism has also been linked to mutations in two principal classes of proteins: those regulating synapse formation and those involved in chromatin remodeling pathways (De Rubeis et al., 2014).

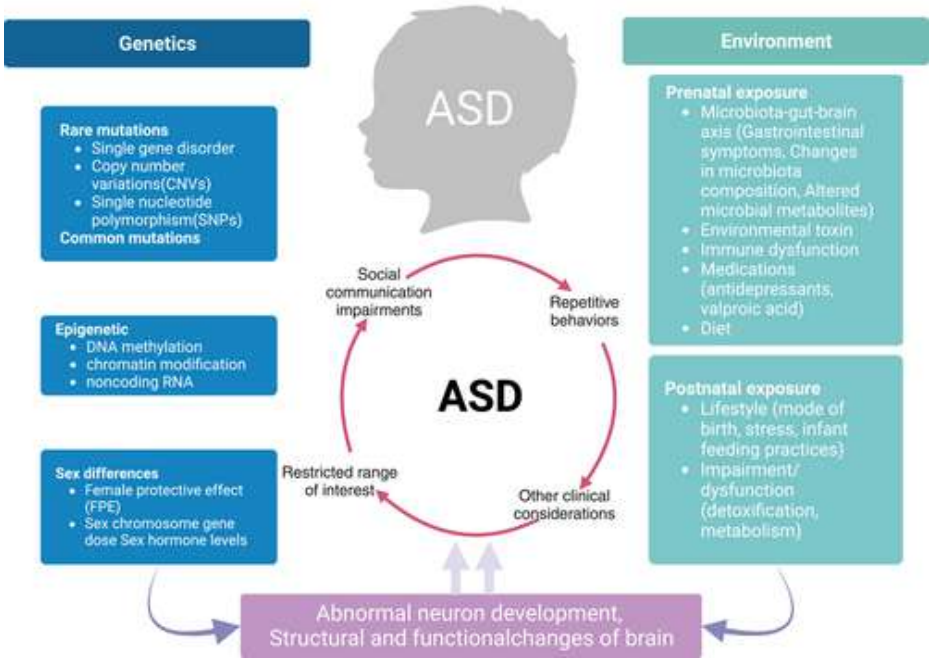

Figure 4. Potential influences of Autism Spectrum Disorder (Zhuang et al., 2024)

Only 10% of autism cases are caused by known genetic mutations and the rest are classified as idiopathic (Betancur, 2011; Zafeiriou et al., 2013). Fragile X Syndrome and Tuberous Sclerosis (TSC) are the most common mutations associated with ASD (Love et al., 2024). Other known genetic forms of ASD account for 1% or less of the autism cases. Some of the causes of genetic mutations causing ASD are:

Copy Number Variations (CNVs): CNVs are duplications, deletions, translocations, and inversions in the nucleotide bases in the chromosomes that can sometimes stretch up to several kilobases. They can either be inherited or be a de novo mutation. A higher percentage of rare CNVs has been reported in ASD-afflicted individuals. They are responsible for around 10% of ASD cases, although there are no known CNVs with complete penetrance (Vicari et al., 2019).

Single Nucleotide Polymorphisms: Around 17-60% of ASD cases are explained by SNPs. The majority of these SNPs are located in the non-coding components of the genome (Golovina et al., 2021).

Epigenetic Alterations: Epigenetics are heritable or acquired changes in the gene expression without any changes to the DNA sequence. These alterations can modify the gene expression level. These alterations occur through four different mechanisms, which are histone modifications, DNA methylation, RNA interference, and RNA modifications (Oztenekecioglu et al., 2021).

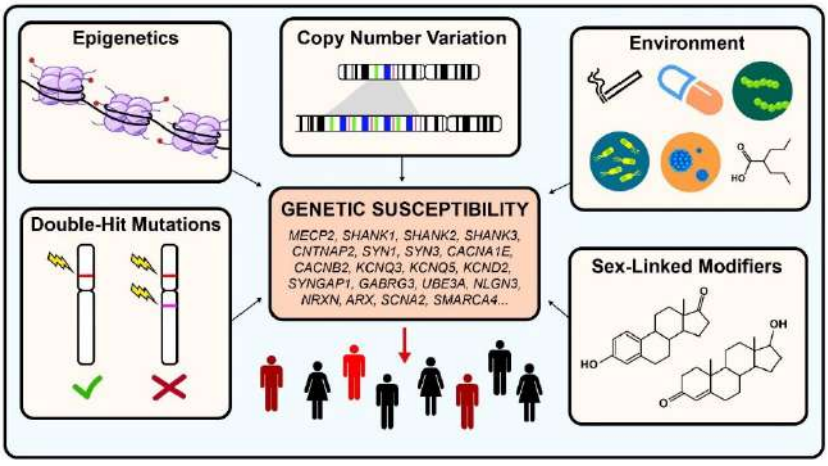
Epigenetics
Copy Number Variation
Environment
GENETIC SUSCEPTIBILITY
MECP2, SHANK1, SHANK2, SHANK3, CNTNAP2, SYN1, SYN3, CACNA1E, CACNB2, KCNQ3, KCNQ5, KCND2, SYNGAP1, GABRG3, UBE3A, NLGN3, NRXN, ARX, SCNA2, SMARCA4...
Double-Hit Mutations
Sex-Linked Modifiers

Figure 5. Modifiers of Autism Spectrum Diorder. Adapted from (Rylaarsdam & Guemez-Gamboa, 2019).

A lot of work has been done to find potential environmental causes of ASD; environmental contaminants, infections during pregnancy, and altered immune response are thought to be linked to an increased risk of autism. During pregnancy, the fetus is partially protected by the placenta which is an imperfect barrier. Hence, during this critical time of development, it is susceptible to viral infections or drugs the mother is exposed to. Non-polar compounds traverse the barrier with ease and have been proven to cause epigenetic changes such as increasing DNA methylation. However, these theories require much more investigation to validate (Love et al., 2024).

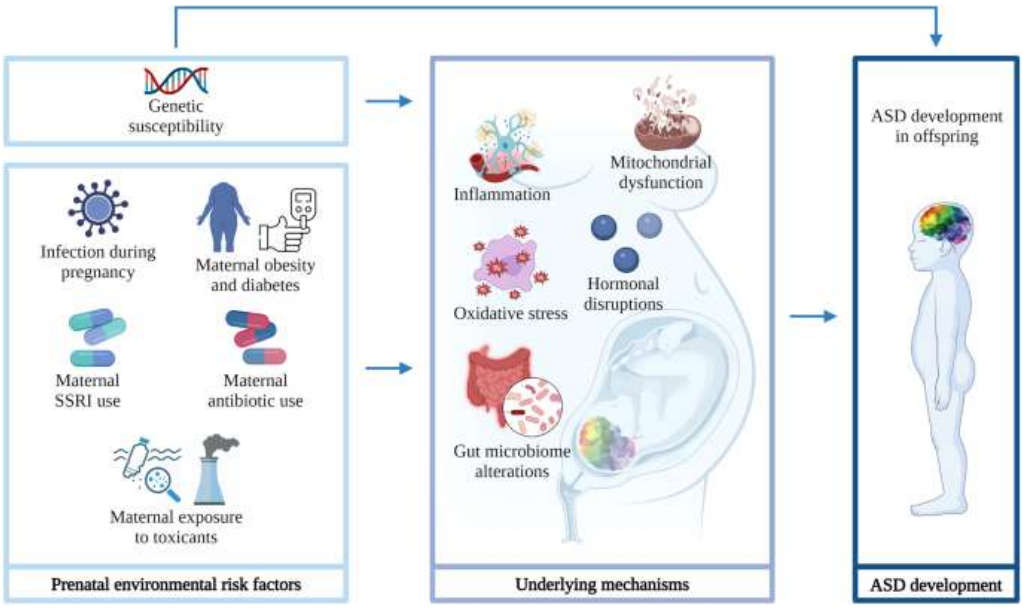

Figure 6.Prenatal environmental risk factors for ASD and their mechanism (Love et al., 2024.)

1.1.3. Prevalence and Diagnosis

In 2021, approximately 61.8 million people, i.e., one in every 127 individuals were on the Autism Spectrum globally (Santomauro et al., 2025). In India, 1 in 68 children are affected by autism which is a huge chunk of the global population considering India is the most populated country (Panda, 2019). Autism is discriminatively more prevalent in males than in females, with there being 4 males afflicted with ASD for every female with the condition (Werling & Geschwind, 2013).

The Diagnostic and Statistical Manual of Mental Disorders, 5th revision (DSM-5), and the International Classification of Diseases, 11th Revision (ICD-11) are the most recent diagnostic systems. DSM-5 combines “Asperger’s disorder”,“ autistic disorder”, “CDD” and “PDD-NOS” under the umbrella of “Autism Spectrum

Disorder" or ASD (Lordan et al., 2021). The symptoms are also divided into two categories them being social communication and rigid or repetitive behaviour. According to DSM-5, a child must meet all three sub-categories within social communication and two out of four within rigid or repetitive behaviour to get a positive diagnosis. Due to the broad definitions, ASD diagnosis occurs with other conditions; motor abnormalities (79%), gastrointestinal problems (~70%), epilepsy (up to 30%), intellectual disability (45%), anxiety (42-56%), depression (12-70%) and sleep disorders (50-80%) are common (Lai et al., 2014).

Symptom Critieria	Traits
Deficits in social communication & social interaction across multiple contexts	Deficits in social-emotional reciprocity; Deficits in nonverbal communication behaviors (e.g., gestures) used for social interaction; Deficits in the development, maintenance, and comprehension of relationships
Restricted, repetitive patterns of behavior, interests or activities	Stereotyped movements, speech, and use of objects; Inflexibility to change of routines or ritualized patterns; Restricted interests with strong, abnormal attachments; Hypo- or hypersensitivity to environmental factors
Symptoms must be present in early developmental period	Symptoms may not manifest until social demands exceed limited capacities
Combination of symptoms significantly impair daily functioning	Level 3 - Requiring very substantial support Level 2 - Requiring substantial support Level 1 – Requiring support

Table 1. DSM-5 criteria for ASD. As adapted from American Psychiatric Association (APA)

Diagnosis is usually done in early childhood with observational tools that measure the child's cognitive and social abilities. The use of these tools is completely dependent on the clinician's judgement, which can lead to both over- and under-diagnosis.

Indian Academy of Pediatrics (IAP) advocates universal ASD screening at 18 and 24/30 months for which M-CHAT-R/F, Trivandrum Autism Behavioral Checklist (TABC), and Social Communication Questionnaire (SCQ) are recommended (National Consultation

Meeting for Developing IAP Guidelines on Neuro-Developmental Disorders under the aegis of IAP Childhood Disability Group and the Committee on Child Development and Neurodevelopmental Disorders et al., 2017).

1.1.4 Brain Structures Implicated in Autism

People with autism spectrum disorder (ASD) have been shown to have structural abnormalities in multiple brain regions, which suggests a widespread disruption in neurodevelopment. There have been reports of hypoplasia and the absence of particular nuclei in the brainstem, which may be a factor in early sensory and autonomic dysfunction. Significant size reductions in the cerebellum, especially in the vermis in lobules VIII–X, are accompanied by a notable 40% increase in cerebellar white matter, suggesting potential dysregulation in motor coordination and connectivity.

The hippocampus and amygdala exhibit altered microarchitecture within the limbic system. This includes increased neuronal packing density in the entorhinal cortex, medial septal nucleus, entorhinal cortex, subiculum, and multiple amygdala nuclei, as well as decreased dendritic branching in the CA1 and CA4 regions of the hippocampus. The hippocampus, amygdalae, and caudate nucleus have all been shown to enlarge in structural imaging studies, while the corpus callosum's cross-sectional area has decreased. Atypical connectivity patterns observed in ASD may be caused by either excessive axon myelination or insufficient dendritic and synaptic pruning, which follow an initial phase of accelerated growth in the cerebral cortex characterized by increased dendritic arborization and synaptogenesis. Additionally, abnormalities in the microstructure of cortical minicolumns, particularly in the frontal and temporal lobes, have been observed, potentially contributing to deficits in social cognition and executive functioning (Polleux & Lauder, 2004).

1.2 The cerebellum and its synapses

1.2.1. Cerebellum Neuroanatomy

The cerebellar circuit is one of the least complex and most studied circuits in the brain. The cerebellar cortex is divided into three layers: the granule cell layer, the Purkinje cell layer, and the molecular layer. The granule cell layer is densely packed with granule cells whose axons project onto Purkinje cell dendrites in the molecular layer. The granule cells are the primary excitatory neurons of the cerebellar cortex and use glutamate or aspartate as their neurotransmitter. At the granular layer, there are also present Golgi cells which have a distinctly larger soma than the granule cells. Purkinje cell (PC) bodies occupy the Purkinje cell layer, they are the sole output from the cerebellum and are inhibitory. The granule cells extend their dendrites to the molecular layer where they form parallel fibres (PFs). Each

parallel fibre synapses onto multiple Purkinje cell dendrites. Every dendrite of the Purkinje cell gets input from approximately 80,000 parallel fibres. Recent studies on the structural organization of cerebellar neuronal networks highlight the role of parallel fibre synapses on Purkinje cells as sites of synaptic plasticity, suggesting their function as a memory mechanism within the cerebellar cortical circuitry. The concept of a *corticonuclear microcomplex*—comprising a localized region of the cerebellar cortex, associated cerebellar and vestibular nuclear neurons, and interconnected inferior olive neurons—has been proposed as a fundamental functional unit of the cerebellum. Experimental evidence, particularly from research on cerebellar modulation of the vestibulo-ocular reflex, supports the hypothesis that such microcomplexes confer precise control over extracerebellar systems, facilitating functions like coordination, predictive regulation, and adaptive learning (Ito, 1984).

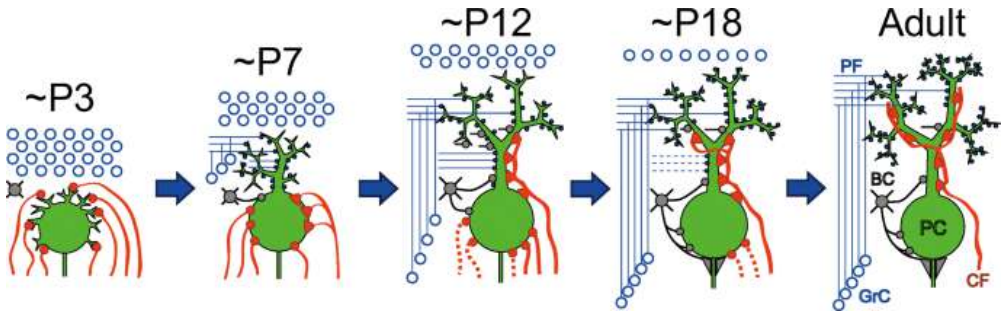

Figure 7. Postnatal Cerebellar synapse development.At P3, multiple climbing fibres synapse onto a single Purkinje cell. At P7, parallel fibres start synapsing onto the dendrites of the Purkinje cell. After PC-PF interaction, a single CF is chosen and rest are pruned. In adults one PC interacts with a single CF. Adapted from (Kano et al., 2018).

Afferent neurons in the cerebellum are of three types: mossy fibres, climbing fibres, and monoaminergic or multilayered fibres. The mossy fibres originate from cell bodies in the cerebellar nuclei and form other nuclei in the spinal cord, medulla, and pons. These fibres use glutamate as a neurotransmitter and thus provide excitatory input to granule and Golgi cell dendrites in the cerebellar glomerulus and the cerebellar nuclear neurons.

The contralateral inferior olive is the source of climbing fibres. These fibres terminate in the molecular layer and entwine with the proximal part of the Purkinje dendritic tree. Normally, one Purkinje cell is innervated by only one CF. This selection happens during development. Initially, multiple CFs (~ five) with equivalent synaptic strength synapse onto the somata of the PCs. Subsequently, one of the CFs becomes stronger during the first post-natal week. Only the chosen CF extends to synapse with the PC dendrites, the rest remain connected only to the soma. Pruning in the second and third post-natal week

eliminates these ‘loser’ synapses. This elimination also requires normal PF-PC synapse formation which occurs around P12 in rodents (Kano et al., 2018).

The PCs project onto three main deep cerebellar nuclei (DCN), the fastigial (medial), interposed, and dentate (lateral) nucleus. In the medial zone, Purkinje cells project to the medial nucleus and further, the projections from the medial nucleus go to the motor cortex via the thalamus. Purkinje cells in the intermediate region synapse onto the interposed nucleus from where the outputs go to the red nucleus and some go to the motor cortex via the thalamus. The majority of outputs from the lateral nucleus connect to areas of the cerebral cortex, mainly motor, somatosensory, and posterior parietal areas via the thalamus.

1.2.2. Cerebellum: A key modulator of motor patterns

The cerebellum processes somatosensory and proprioceptive signals originating from the peripheral nervous system, along with extensive input from various regions of the cerebellar cortex. It plays a key role in modulating motor patterns and integrating sensory and internal feedback. Although the precise role of the cerebellum in gait coordination remains unclear, gait ataxia is one of the most characteristic signs of cerebellar damage.

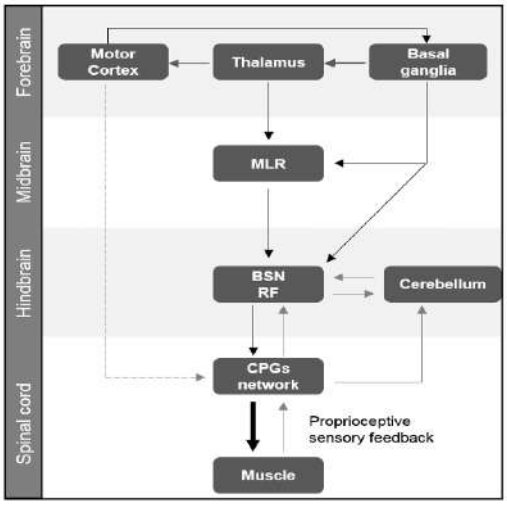

Figure 8. Circuits involved in Locomotion.

Illustration (Figure 8.) of neuronal structures and pathways involved in basic rhythmic activities like locomotion**.** Higher brain regions—including the hindbrain, midbrain, and forebrain—are responsible for initiating, planning, and adjusting locomotor activity. Within the spinal cord, central pattern generators (CPGs) produce the rhythmic motor patterns required for movement. Motor cortex inputs (represented by dotted arrows) help fine-tune and trigger motor actions. Solid black arrows depict direct command pathways, while grey arrows represent feedback circuits. Abbreviations: MLR – mesencephalic locomotor region; BSN – brainstem nuclei; RF – reticular formation; CPGs – central pattern generators. Adapted from (Goulding, 2009).

Interlimb coordination and temporal coordination worsening are the hallmarks of cerebellar ataxia (Morton & Bastian, 2007; Palliyath et al., 1998). A slower gait with a widened base of support and an erratic limb trajectory is observed in cerebellar lesion patients (Earhart & Bastian, 2001). Excessive hip and knee flexion on the affected side with an overly high swing phase and then abrupt lowering is observed. Trajectory of walking is also severely affected and patients have a difficult time turning or stopping during fast movement. Additionally, the cerebellum is also involved in motor learning (Raymond et al., 1996). Eyelid conditioning experiments in mice are an example of simple forms of cerebellum-dependent motor learning (De Zeeuw & Yeo, 2005).

1.2.3. Cerebellar aberrancies in autism

A plethora of cerebellar abnormalities have been seen in ASD patients. Reductions in the size of the cerebellum including the vermis, lobules VI, and VII were seen in structural MRI studies in ASD patients. Enlargement in cerebellar white matter and a reduced grey/white matter ratio is also observed. Over-connectivity in the cerebro-cerebellar connections has been reported using resting state Functional Connectivity in children and young adults with ASD. A reduction in number of Purkinje cells has been seen. Neurochemical levels are also disrupted in ASD cases. Postmortem studies of individuals with autism have consistently shown a reduction in the expression of glutamic acid decarboxylase 67 (GAD67) mRNA in Purkinje cells (PCs) of the cerebellum. GAD67 is a key enzyme involved in the synthesis of GABA, the brain's primary inhibitory neurotransmitter. Interestingly, an increased expression of GAD67 mRNA has been observed in cerebellar molecular layer interneurons, possibly indicating a compensatory response to disrupted inhibitory signaling from PCs to the deep cerebellar nuclei (DCN). Additionally, within the DCN, a subset of larger GABAergic neurons that specifically project to the inferior olive (IO) show reduced expression of GAD65 mRNA, another enzyme critical for GABA synthesis (Mapelli et al., 2022). These alterations in GABAergic neurotransmission may disrupt the normal oscillatory activity of the IO, which plays a crucial role in regulating the precise timing of PC firing. Since the cerebellum is integral to motor coordination and timing, such disruptions could contribute to motor impairments and atypical locomotor patterns commonly observed in individuals with autism.

1.3. Neurexin1 KO Model

1.3.1. Neurexin family of genes

Neurexins are a class of presynaptic cell adhesion proteins. Multiple genes, *Nrxn1, Nrxn2, and Nrxn3* have three alternative promoters α, β, γ. In mammals, a long α- Neurexin pre-

mRNA is formed and a shorter β- Neurexin pre-mRNA is formed which codes for 1500 and 450 amino acids respectively.

They serve as presynaptic receptors for a wide range of structurally diverse extracellular binding partners because of the widespread alternative splicing that results in distinct isoforms linked to particular neuronal cell types. This implies that they serve as a focal point for presynaptic structure organization. A variety of binding partners, such as the secreted protein Cerebellin 1 (CBLN1), the transmembrane proteins Neuroligin 1–4, α-Dystroglycan, the Leucine-rich repeat transmembrane proteins (LRRTM1–4), and Calsyntenin 3, bind to neurexins as presynaptic receptors.

Neurexin1 is a single-pass type I membrane protein encoded by the NRXN1 gene. *Nrxn1* has 24 exons and is 1.1 MB large, one of the largest human genes. Mutations in Neurexin genes particularly the Neurexin1 gene have shown significant risk for various neurodevelopmental, psychiatric, and neurophysiological phenotypes as is seen from human genetic studies.

1.3.2. Neurexin in autism:

The initial identification of two rare missense mutations (p.S14L and p.T40S) in exon 1 of the *NRXN1β* gene—discovered after analyzing all three β isoforms in 131 Caucasian and 61 African-American individuals with ASD—alongside their absence in over 500 control subjects, strongly implicated NRXN genes in autism spectrum disorder (ASD). In a pivotal study, the Autism Genome Project Consortium investigated 1,181 families, each with at least two members diagnosed with ASD, and discovered a ~300 Kb hemizygous deletion within the coding region (2p16.3: 50,430–50,785) of the *NRXN1α* isoform in two siblings. Building on this finding, Kim et al. reported a structural disruption in intron 5 of *NRXN1α* in one ASD patient, and a chromosomal translocation located approximately 750 Kb upstream of the 5′ region of the same isoform in another unrelated individual (Kim et al., 2008a).

Genetic variations within the 3' untranslated region (3' UTR) of the NRXN1 gene have been linked to a moderate increase in the risk of developing Autism Spectrum Disorder (ASD) and schizophrenia, even in individuals without clinical symptoms. Mutations in Neurexin1 account for approximately 0.5% of ASD cases (Kim et al., 2008b). Interestingly, copy number variations (CNVs) specifically affecting the NRXN1α isoform—rather than NRXN1β—have been more strongly associated with both ASD and schizophrenia. In rare instances, disruptions in NRXN1α have been correlated with

significantly reduced intellectual functioning, underscoring the potential severity of these genetic alterations.

Neurexin1α KO mice exhibit impaired Pre-pulse Inhibition (PPI), indicating aberrant sensorimotor gating which is also seen in ASD patients. It has also been observed that they show enhanced motor learning as indicated by rotarod experiments. Increased frequency of grooming bouts has also been observed (Etherton et al., 2009). Although Neurexin1α KO mice demonstrated completely normal social behaviour, Neurexin1α KO rats have been known to show social deficits, the deficits were more severe in the homozygous animals compared to the heterozygous ones (Achterberg et al., 2025; Esclassan et al., 2015).

1.3.3. Neurexin in cerebellum

In cerebellar granule cells, NRXN knockdown also inhibits GluRδ2's synaptogenic activity. PF-PC synapse formation in the cerebellum is mediated by the trans-synaptic interaction of postsynaptic GluRδ2 in Purkinje cells and presynaptic NRXNs via extracellular scaffolding protein present in granule cells. Mutations in any one of the components impair presynaptic differentiation and severe reduction in synapse assembly and density is observed. Previous studies have shown that GluRδ2 preferentially interacts with Neurexin (NRXN) isoforms containing the S4 insert, while Neuroligins (NLGNs) tend to bind isoforms lacking S4. Notably, RT-PCR analyses have indicated that S4-containing NRXN variants are predominantly expressed in the cerebellum, with S4-lacking forms being barely detectable, whereas both variants are present in the cortex and hippocampus. Although my study does not directly examine these molecular differences, this region-specific expression pattern provides a rationale for focusing on cerebellum-related behavioural phenotypes in Neurexin1 knockout rats (Uemura et al., 2010).

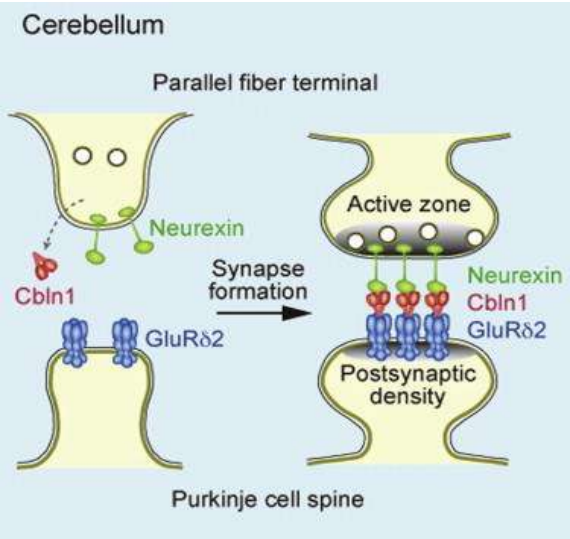

Figure 9. Pre-synaptic neurexin interacts via Cbln1 with post-synaptic GluD2 to mediate Purkinje cell-Parallel fiber synapse formation. Adapted from Uemura et al., 2010.

Chapter 2: lncRNA in Cerebellar Synapses in ASD Rat Model

2.1. Introduction

2.1.1. Long Non-Coding RNA (lncRNA)

Long Non-Coding RNA (lncRNA) have been described as non-coding transcripts that are more than 200 nucleotides (200nt) long and coded by Polymerase II. However, recently it has been suggested that the categorization be changed to more than 500 nt long (Mattick et al., 2023). Despite their low abundance, exhibit high specificity to its targets. (Jachowicz et al., 2022). They constitute a substantial portion of the genomes of complex organisms and are typically expressed in a cell-type-specific manner. Structurally, lncRNAs share several characteristics with mRNAs: they are usually transcribed by RNA polymerase II, possess canonical splice junctions (GU/AG), exhibit comparable intron and exon lengths, undergo alternative splicing, and may be either polyadenylated or non-polyadenylated. They also display similar chromatin signatures, marked by the same histone modifications as protein-coding genes.

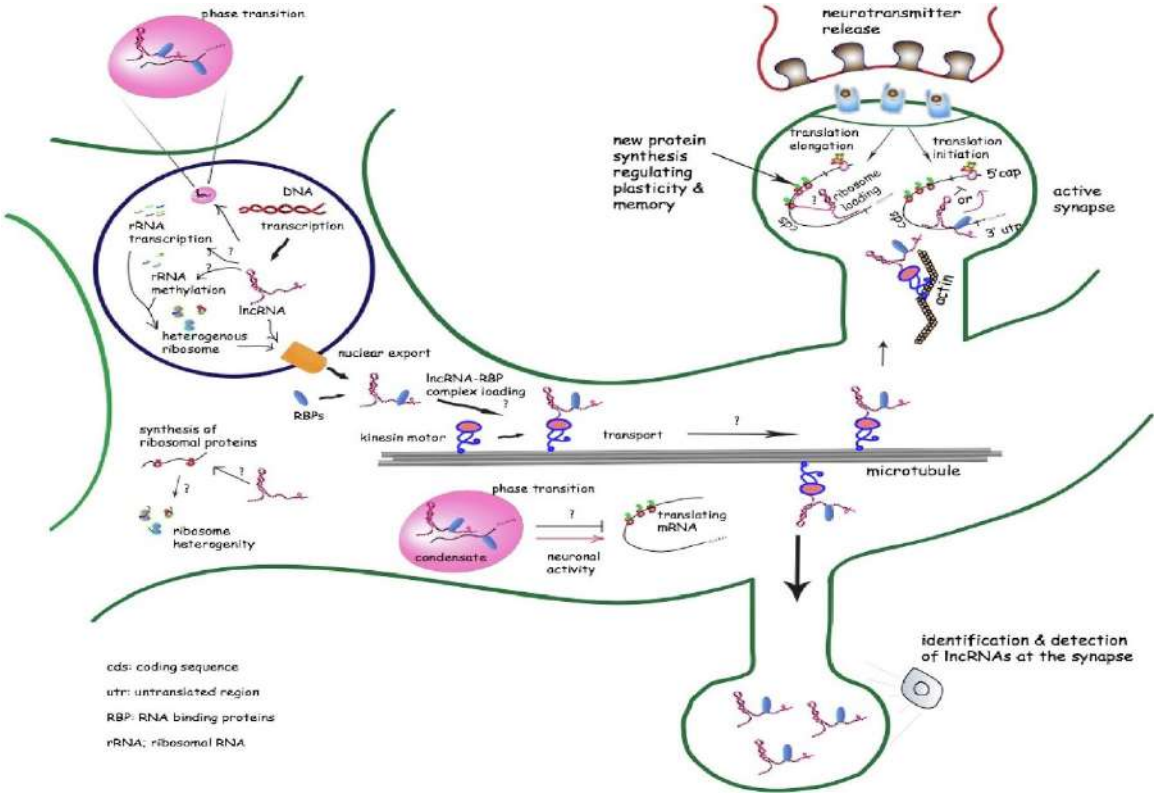

Figure 10. Subcellular localization and various functions of lncRNAs (Samaddar & Banerjee, 2021).

In the nervous system, the function of lncRNAs is closely linked to their subcellular localization. Nuclear lncRNAs regulate gene expression and chromatin architecture, thereby influencing

essential processes such as neuronal differentiation and synaptogenesis. For example, TUNA and RMST guide stem cells toward neural lineages by interacting with transcription factors like Sox2. Similarly, Evf2 plays a crucial developmental role by regulating genes essential for the formation of GABAergic interneurons—cells that are vital for maintaining the brain's excitatory-inhibitory balance, which is frequently disrupted in neurodevelopmental disorders including autism spectrum disorder (ASD) and schizophrenia.

Other nuclear lncRNAs, such as neuroLNC and MALAT1, are responsive to synaptic activity and regulate the expression of genes involved in synapse function, neurotransmitter release, and dendritic morphology. Gm12371, for instance, contributes to shaping dendritic spine architecture, which is a key structural component of synaptic plasticity and learning (Clark & Blackshaw, 2017).

Importantly, most mutations that affect phenotypic traits occur in non-coding regions, rich with genes expressing lncRNAs (Hon et al., 2017). This makes lncRNAs attractive candidates for investigating the molecular underpinnings of ASD. Given their role in brain development, synaptic function, and gene regulation, disruptions in lncRNA expression or function may contribute to the atypical neural connectivity and signaling observed in individuals with autism.

2.2. Aims and Objectives

Identify differential expression of lncRNAs in the cerebellar synapse of Neurexin1 KO ASD rat model.

- Prepare Synaptoneurosome for wild-type and Neurexin1 KO rats.
- Isolate protein and perform protein estimation using BCA assay.
- Validate Synaptoneurosome by western blot.
- Perform RNA isolation to analyze differential expression through qPCR analysis.

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2.3. Material and Methods:

2.3.1. Animals

SD-Nrxn1em1 ZFN mutant rat strain and Wild Type Sprague Dawley rats were used. All animals were issued from the National Brain Research Centre (NBRC) animal house facility from the protocol numbers NBRC/IAEC/2015/186 as approved by IAEC (Institutional Animal Ethics Committee) of NBRC registered with the CPCSEA (Committee for Control and Supervision of Experiments on Animals) (Registration number: 464/GO/ReBi-S/Re L/01/CPCSEA).

For this study, male SD-Nrxn1 KO and wild-type Sprague Dawley rats (p70-p90) were used, which were kept in a group of 3-4 rats per cage under a 12-hour light/dark cycle at a temperature ranging from 22-24°C, with a relative humidity maintained between 40-45% and had ad libitum access to both water and food. The mice were sourced from the animal facility at the National Brain Research Centre and all experimental procedures were carried out during the light phase per the guidelines and regulations established by the Institutional Animal Care and Use Committee of the National Brain Research Centre.

The SD-*Nrxn1*^{em1} strain was produced by Sage Laboratories by injecting zinc finger nuclease targeting rat Nrxn1 into Sprague Dawley embryos. They possess a 16bp deletion resulting in the knockout of the Neurexin1 gene. One homozygous animal and a wild-type Sprague Dawley rat are kept in breeding pairs at the age of p50-p60. At p21, pups are weaned, separated by gender, and kept in groups of 3-4.

2.3.2.Genotyping

Materials Required

1. 500mM NaOH (prepared in autoclaved MQ)
2. 1M Tris (pH =7.4)
3. Microcentrifuge Tube
4. Heat Block
5. Table top Vortex
6. Table top centrifuge
7. Pipettes and tips
8. Proflex PCR machine
9. Reagents for PCR
    - 9.1. 10X PCR Buffer with MgCl2
    - 9.2. 2mM dNTP mixture

9.3. Neurexin1 KO forward and reverse primer

9.4. DMSO

9.5. Taq Polymerase

9.6. UltraPure Water

10. Regents for agarose gel electrophoresis

10.1 Agarose Powder

10.2 1X Tris-acetate-EDTA buffer: 1L of 50X buffer contains 242g tris base (Merck), 57.1 ml glacial acetic acid, and 100 ml 0.5M EDTA solution (pH 8.0).

10.3 Ethidium Bromide Solution (10mg/ml)

10.4 100bp Ladder (6 microlitres contains 0.5 micrograms of DNA)

11. Bromophenol Blue Solution: 5ml of 6X Loading Buffer contains 1.5ml glycerol, 0.0125g bromophenol blue, and 0.0125g xylene cyanol FF.

12. BioRad Electrophoresis Tank

Methodology

a) Tail Cutting: Weaned rats at p21 are separated based on gender and ear tagged. 0.5mm piece of their tail is collected in aseptic conditions.
b) Tail Digestion: 500 microlitres of 50mM NaOH is added to the cut tails and boiled for 45 mins. After vortexing and cooling, 50 microlitres of 1M Tris (pH 7.4) is added and the sample is boiled again for 2 minutes. After centrifugation, the supernatant is transferred to a separate Eppendorf.
c) Polymerase Chain Reaction (PCR): PCR samples are prepared as follows:

Reagent	Required concentration	Volume (in microlitres)
1. 10X PCR Buffer + MgCl2	1X	1
2. 2mM dNTPs	0.2mM	1
3. 10µM Forward Primer	0.4µM	0.4
4. 10µM Reverse Primer	0.4µM	0.4
5. DMSO	0.4%	0.04
6. DNA template		2
7.G9 Taq Polymerase (2.5U/µL)	0.5U	0.2
8. Nuclease Free Water (NFW)		4.96
TOTAL		10,000

Table 2. PCR Reaction Composition

D) Agarose Gel Electrophoresis:

- The PCR products are stained with 0.5 microlitres of Bromophenol Blue Loading Buffer.
- 5 microlitres of 100bp ladder and the PCR products are loaded in 3% Agarose Gel made in TAE.
- The gel is run at 120V for 60 minutes until the loading dye has run 3/4th the length of the gel.
- The gel is imaged under UV light.

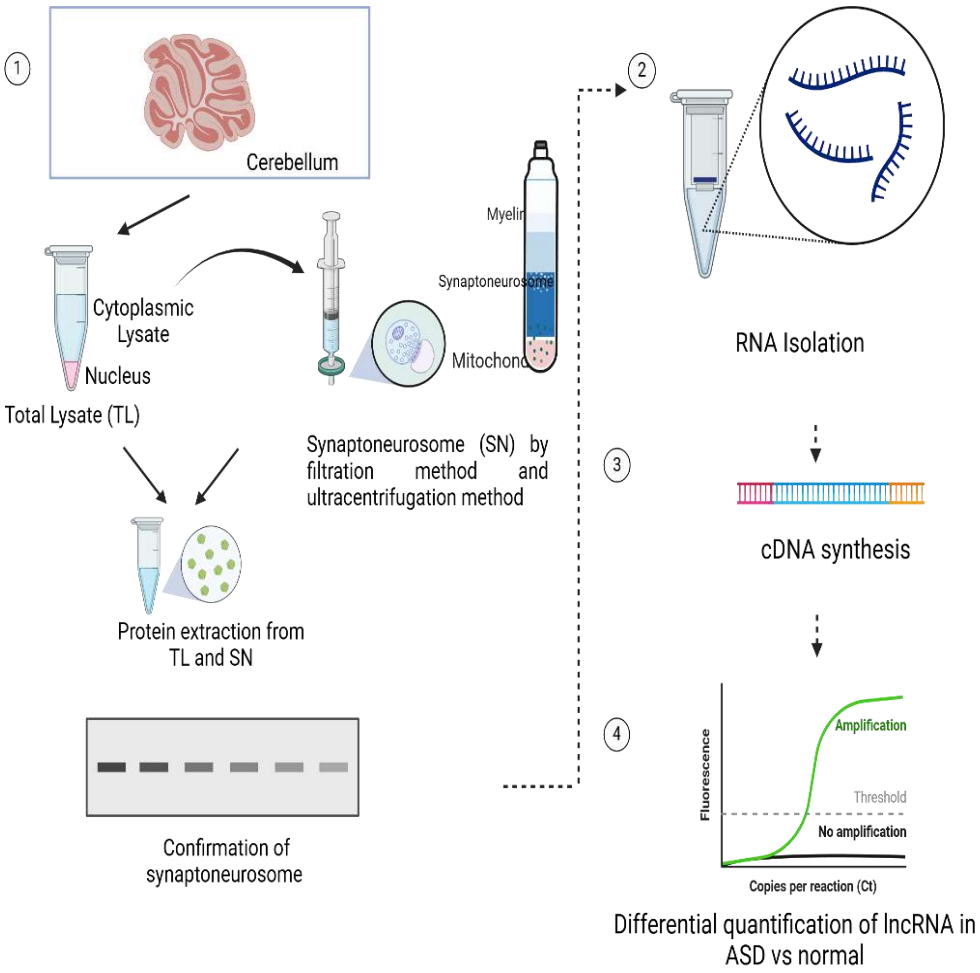

Figure 11. Synaptoneurosome Fraction preparation for identifying differential expression of lncRNA.

2.3.4. Synaptoneurosome Preparation through Filtration Method

Material Required:

1. Autoclaved Homogenizer
2. Takara RNase Inhibitor
3. Microcentrifuge Tube
4. Synaptoneurosome buffer
5. 1x Phosphate Buffered Saline (PBS)
6. 30G Insulin syringe (1ml)
7. 18G syringe (2 ml)
8. Filter (100 and 5 microns)
9. CO2 cylinder
10. Micropipettes and tips
11. Sterile surgical blade

12. Scissors
13. Forceps

Synaptoneurosome buffer			
S.no	**Reagents**	**Required Molarity**	**Required volume**
1.	1M Tris (ph. 7.4)	5mM	25µl
2.	1M $CaCl_2$	2.2 mM	11 µl
3.	200 mM Na_2HPO_4	0.5mM	12.5 µl
4.	200 mM KH_2PO_4	0.4mM	10 µl
5.	1M $NaHCO_3$	4mM	20 µl
6.	5M NaCl	80mM	80 µl
7.	Ultrapure water		4.84 ml
Total volume			5ml

Table 3. Composition of synaptoneurosome buffer

Methodology

1) Animals were euthanized promptly using 20 liter/min carbon dioxide (CO2) and cervical dislocation, following the isolation of the cerebellum.
2) 5 µl of 40U/ml RNase inhibitors (#Takara) were added to the 5 ml SN buffer
3) The isolated cerebellum was subjected to homogenization using 1000 µl of SN buffer in a Microcentrifuge Tube (1.5 ml), with a total of 18-20 up-and-down strokes performed with a sterile homogenizer.
4) Subsequently, an additional 1000 µl was introduced into the homogenate, which was then passed through a 1 ml insulin syringe until the lysate passed through without any resistance.
5) 10% of the total lysate volume was kept for western blotting and RNA isolation.

6) The remaining 85% of the homogenate was transferred to a 2 ml syringe (18 gauge) and filtered through a 100 µ filter, resulting in the formation of the first filtrate, F1.
7) F1 underwent another filtration through a 5 µ filter to generate F2.
8) Following this, F2 was centrifuged at 10,000 xg for 20–30 minutes at 4 °C, and the resulting pellet was resuspended in 20 µl SN buffer.
9) The resuspended pellet was then stored at -80 °C for subsequent analysis.

2.3.5. Bicinchoninic acid (BCA) assay

Materials required

1. Tecan microplate reader
2. Incubator
3. Bovine serum albumin (2mg/ml)
4. Thermo scientific BCA kit
5. 1x Tris buffer saline (TBS)
6. 96 well plate
7. Aluminum foil

Methodology

Standard preparation

Bovine Serum Albumin (BSA) has been used to prepare standards. The concentration of BSA is 2 mg/mL. From that, different known concentrations such as 800, 400, 200, 100, 50, 25, and 12.5 µg/mL have been prepared using the serial dilution method given below. For the blank, the BSA was not added; only the diluting 1x tris buffered saline was used.

Standard	BSA volume	1x TBS	Concentration
A	52 µl of 2mg/ml BSA solution	78 µl	800 µg/ml
B	65 µl from A	65 µl	400 µg/ml
C	65 µl from B	65 µl	200 µg/ml
D	65 µl from C	65 µl	100 µg/ml
E	65 µl from D	65 µl	50 µg/ml
F	65 µl from E	65 µl	25 µg/ml
G	65 µl from F	65 µl	12.5 µg/ml
H	-	65 µl	0

Table 4. Preparation of BSA standards for BCA.

Samples preparation

- The Synaptoneurosome (SN) and Total Lysate (TL) samples were each diluted by a factor of 1/50 and 1/100.

Samples	**Synaptoneurosome**	**Total Lysate**
1/50 dilution	Sample:2.4 µl 1x TBS: 117.6 µl	Sample:2.4 µl 1x TBS: 117.6 µl
1/100 dilution	60 µl from 1/50 dilution 1x TBS: 60 µl	60 µl from 1/50 dilution 1x TBS: 60 µl

Table 5. Preparation of samples for BCA

Working Solution Preparation:

The working BCA solution was prepared by combining solutions A and B from the Thermo Scientific kit in a 50:1 ratio.

1. 25 microlitres of diluted samples (1/50 and 1/100 concentrations) were added to the wells of a 96-well plate.
2. Then, 200 µl of the prepared BCA working solution was added to each well containing the samples.
3. The plate was covered with aluminum foil and incubated at 37 °C in a bacterial incubator. After incubation, the optical density (OD) was measured using a Tecan microplate reader.
4. Based on the OD readings, a standard curve was generated to determine the Y-values, which were then used to calculate the protein concentration (µg/µl).

2.3.6. Western blotting

Materials required

1. Nitrocellulose membrane
2. SDS page: Protein running and transfer set
3. Ponceau S stain
4. 30% Acrylamide and Bis-acrylamide (w/v) (29:1 ratio): 87 g of acrylamide and 3 g of bis-acrylamide in a total of 300ml. Store at 4°C.
5. 5% stacking gel (3 ml): sterilized Milli-Q water (2.1 ml), 30% Acrylamide and Bis-acrylamide (0.5 ml), 1M Tris-HCL (pH 6.8) (0.38 ml), 10% sodium dodecyl sulfate (SDS) (30 µl), 10% ammonium persulfate (30 µl), N, N, N′, N′-Tetramethylethylenediamine (TEMED) (3 µl).
6. 10% Resolving Gel (20 ml): sterilized Milli-Q water (7.9 ml), 30% Acrylamide and Bis-acrylamide (6.7 ml), 1.5 M Tris-HCL (pH 8.8) (5 ml), 10% sodium dodecyl sulfate (SDS) (200 µl), 10% ammonium persulfate (200 µl), N, N, N′, N′-Tetramethylethylenediamine (TEMED) (8 µl).

7. 1M Tris-HCL (pH 6.8): 1.21 g of tris base dissolve in autoclaved Milli-Q water (7 ml) and make the volume total 10 ml after dissolved. After adjusting the pH to 8.8, carefully add drop by drop of HCL.

8. 5 M Tris-HCL (pH 8.8): Tris base (1.81 g) dissolves in autoclaved Milli-Q water and makes the volume 10 ml. Adjust the pH to 8.8 by carefully adding HCl.

9. 10X Tris-glycine (1L): Add Tris base (30.2 g) and dissolve in 500 ml of autoclaved Milli Q water. After, add 144 g of glycine batch-wise, dissolve it overnight, and make up the volume to 1L. Store at room temperature (RT).

10. Transfer buffer (1L): 10X Tris-glycine, 100 ml; methanol, 200 ml; autoclaved Milli-Q water, 700 ml. Store at 4°C.

11. Running buffer (1L): 10X Tris-glycine, 100 ml; 10% SDS, 10 ml; autoclaved Milli-Q water, 890 ml. Store at RT.

12. 10% SDS: The total volume of the 100-ml solution contains 10 g of SDS; dissolve and make the volume up to 100 ml with autoclaved Milli-Q water.

13. Tris-buffered saline (TBS) pH 7.4: 1 liter of 10X TBS consists of Tris (24 g) and NaCl (88 g) and dissolves in sterilized MilliQ water until the quantity reaches 1L. Produce 1X TBS before utilization by incorporating 10 ml of 10X TBS and 90 ml of MilliQ water.

14. TBST: 1 liter of 1x TBST composed of 10x Tris-buffered saline (TBS) pH 7.4 (100 ml), 0.1% Tween 20 (1 ml), milli-Q water (899 ml)

15. 5% bovine serum albumin (BSA): A total solution of 50 ml contains 2.5 g of BSA. Dissolve in 1x TBST and make the volume up to 50 ml.

16. Primary antibody (mouse); GFAP 1.5: 1000 dilution; PSD95 1:1000; Histone1 1:500 dilution in 5% BSA

17. Primary antibody (rabbit); Synapsin 1:1000 dilution in 5% BSA

18. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1:5000 dilution anti-mouse and anti-rabbit): A total antibody solution of 5 ml contains 1 µl of the secondary antibody and 4,999 µl of 5% BSA.

19. Substrate for HRP: The substrate comprises equal volumes of luminol and hydrogen peroxide in a 1:1 ratio of milli-Q water and luminol-hydrogen peroxide. The total HRP substrate requires a volume of 2 ml; luminol is 0.5 ml; hydrogen peroxide is 0.5 ml; and milli-Q water is 1 ml.

20. 6x Lamelli buffer total volume of 20 ml contains 7 ml of 1 M Tris HCl, pH 6.8, 2g of SDS, 6 ml of glycerol, 1.86 g of DTT, 2.4 mg of bromphenol blue, MilliQ up to 20ml.

21. Bio-Rad ChemiDoc imaging system

22. IOLIGHT LIMITED Fluorescent Microscope

23. METTLER TOLEDO pH meter

24. Rocker

Methodology

1. The samples were made in Lamelli buffer and heated to 95°C.

2. 1 µg of protein was used.

3. The samples were loaded and were resolved on a polyacrylamide gel at 70 V for stacking and 100 V for resolving.

4. The proteins from the gel were wet transferred onto a nitrocellulose membrane at 65 volts for 90–120 minutes following which, they were visualized using Ponceau S staining.

5. Subsequently, the membrane was blocked using 5% BSA for 3 hours at room temperature.

6. For immunoblotting, the nitrocellulose membrane was incubated with primary antibodies for GFAP (1:2000 dilution), PSD95 1:5000, and synapsin1 overnight at 4°C.

7. Following the overnight incubation, the membrane was washed eight times with 1x TBST for 10 minutes each.

8. The membranes were then exposed to secondary antibodies (Goat Anti-Mouse, dilution 1:5000 for PSD95, GFAP, Goat Anti-Mouse, dilution 1:10000 for tuj1 and Goat Anti- dilution 1:5000 for synapsin1) and were incubated for 3 hours at room temperature.

9. Six washes with 1x TBST for 10 minutes each were performed.

10. Finally, the results were visualized using a Chemi-Doc after adding the substrate.

2.3.7. RNA Isolation

Materials required

1. Trizol reagent
2. Chloroform
3. Isopropanol
4. 100% Ethanol
5. Ultrapure water (Invitrogen)
6. Refrigerated centrifuge

Methodology

1. To the 100 µl of the sample (synaptoneurosome and total lysate separately), 250 µl of Trizol was added and thoroughly mixed while on ice.

2. The mixture was then centrifuged at 8000 x g for 5 minutes at 4 °C. The supernatant was transferred to a distinct tube, and an additional 250 µl of Trizol was incorporated and resuspended.

3. To the Trizol-supernatant mixture, 250 µl of chloroform was added. The mixture was inverted 6–10 times to mix thoroughly and incubated on ice for 2 minutes.

4. Following incubation, the mixture was centrifuged at 18,000 xg for 15 minutes at 4 °C.

5. After centrifugation, three phases – the aqueous, interphase, and organic phases, were developed. The aqueous phase was carefully separated into another tube without disturbing the interphase.

6. Subsequently, 250 µl of isopropanol was introduced to the aqueous phase, and the solution was incubated overnight at -80 °C.

7. The mixture was then centrifuged at 18,000 xg for 2 hours at 4 °C.

8. After centrifugation, the supernatant was discarded, and the pellet was washed with 70% ethanol and centrifuged again at 18,000 x g for 1 hour at 4 °C.

9. Ultimately, the pellet was desiccated and resuspended in 20 µl of ultra-pure water (Invitrogen).

2.3.8. DNase Treatment

Materials required

1. DNase Enzyme (Turbo DNase kit Invitrogen, catalog number: AM2238; 2U/µL)
2. DNase buffer (Turbo)
3. Inactivating reagent (Turbo)
4. Nuclease free water
5. Water bath
6. Refrigerated centrifuge

Methodology

1. After the RNA isolation, the isolated RNA was treated with DNase to avoid any contamination of DNA in the sample. The sample was treated with the DNase enzyme with the reaction mixture given below, following the protocol.

   Reaction mixture

   Table:

Sample (volume)	10X Turbo DNase Buffer	Turbo DNase Enzyme (2U/ µl)	Ultra-Pure water	Total reaction volume
9.5 µl	1.2 µl	0.5 µl	0.8 µl	12 µl

Table 6. Reaction mixture for DNase treatment

2. The reaction mixture was then incubated in a water bath for 30 minutes at 37 °C.
3. After the incubation, 1 µl of the inactivating reagent was added to the mix and incubated at room temperature for 5 minutes.

Following that the mixture was centrifuged at 10,000 xg, 10 mins for 4 °C, and the supernatant was isolated without disturbing the pellet and performed nanodrop to determine the RNA concentration (µg/µl).

2.3.9. cDNA preparation

Materials required

1. Thermal cycler
2. Random Hexamers (50ng/µL)
3. Ultrapure water
4. 10mM Deoxynucleotide triphosphate (dNTPs) Mix
5. 10x Reverse Transcription buffer
6. 25mM Magnesium chloride (Mgcl2)
7. 0.1 M Dithiothreitol (DTT)
8. RNase Out (RNase inhibitor)
9. Reverse transcriptase enzyme (Superscript III)
10. RNase H enzyme

Methodology

Step 1: Denaturation (RNA dimers or self-ligation is denatured)

- For this step, the mixture containing the sample, primer, and dNTPs was incubated for five minutes at 65 °C in a thermal cycler. Following that, the mixture was kept on ice for 1 minute.

- After the incubation, a reverse transcription mix was made, added to the sample, and 1 cycle in the thermal cycler to synthesize the cDNA as per the given conditions below.

Sample	10mM dNTPs	Random Hexamers	Ultra-pure water	Total volume
3µg of sample	1 µl	1 µl	Upto 10 µl	10µl

Table 7. Reaction mixture for denaturation of RNA dimer

Reaction condition

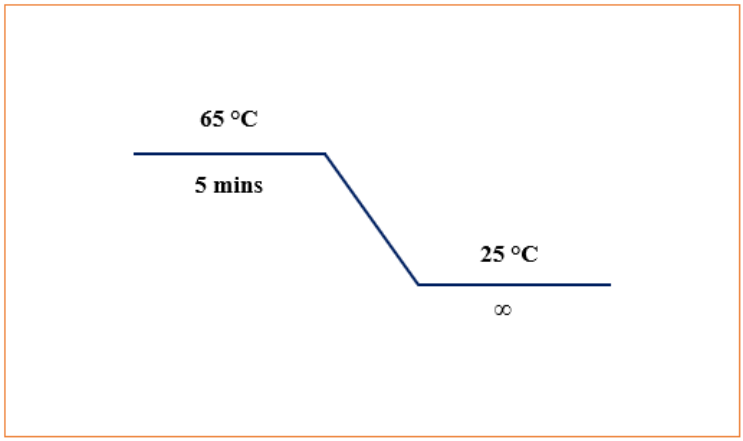

Figure 12.Reaction condition for denaturation of RNA dimer

Step 2: Synthesis mix

Components	**Required Molarity**	**Volume (µl)**
10x RT buffer		2
25mM Mgcl2	11.11mM	4
0.1 M DTT	22mM	2
RNase Out (RNase inhibitor; 40U/µL)	20U	0.5
Superscript III (Reverse transcriptase; 200U/µL)	100U	0.5
Total		9 µl

Table 8. Reaction mixture for reverse transcription

PCR incubation (1 cycle)

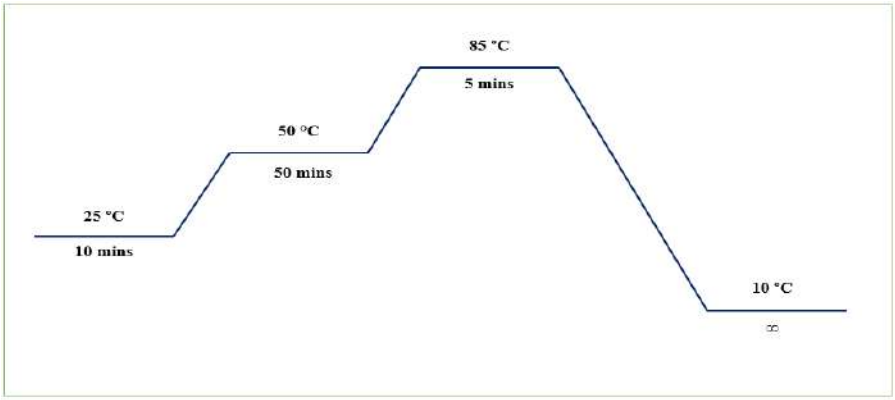

Figure 13.Reaction condition for reverse transcription

Step:3 Removal of RNA

- Following the cDNA synthesis, to remove the RNA from the cDNA sample, 0.5 µl of RNase H (2U/µL) is added to the mixture and incubated at 37 °C for 30 minutes in a thermal cycler.

Reaction condition

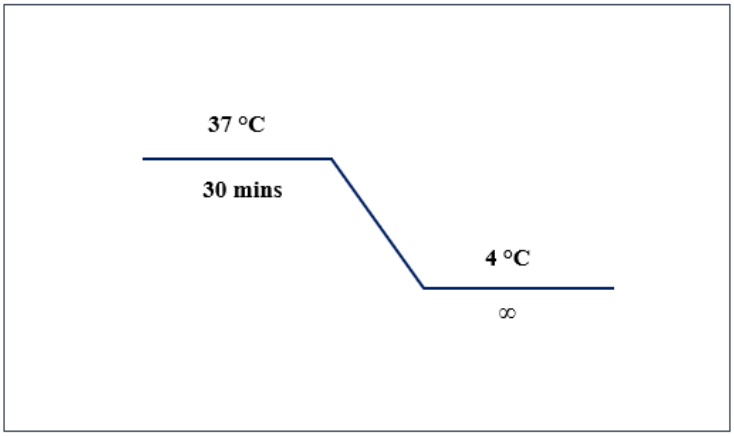

Figure 14. Reaction condition for RNase treatment

2.3.10. Quantitative Polymerase Chain Reaction (qPCR)

Materials required

1. Sybr green master mix (Applied Biosystems PowerUp SYBR Green Master Mix for qPCR)
2. Forward and reverse primer (18s rRNA, Gas5 and Pvt1)
3. cDNA sample

4. Nuclease free water
5. qPCR machine

Methodology

1. To quantify lncRNA expression levels in both total lysate and synaptoneurosome fractions from the cerebellum of postnatal day 70 (P70) Sprague Dawley wild-type and heterozygous *Neurexin1* knockout rats, quantitative PCR (qPCR) analysis was performed using synthesized cDNA derived from extracted RNA.

2. The qPCR reaction mixture consisted of SYBR green, forward and reverse primers, cDNA sample, and nuclease-free water in specified quantities as outlined. Subsequently, the reaction underwent a cycle in qPCR as given by the conditions below.

S.no	Components	Required volume
1.	Sybr green mixture	5 µl
2.	Forward and reverse primer	1 µl
3.	cDNA sample	1 µl (30 ng)
4.	Nuclease free water	Upto 10 µl
Total volume		10 µl

Table 9. Reaction mixture for qPCR

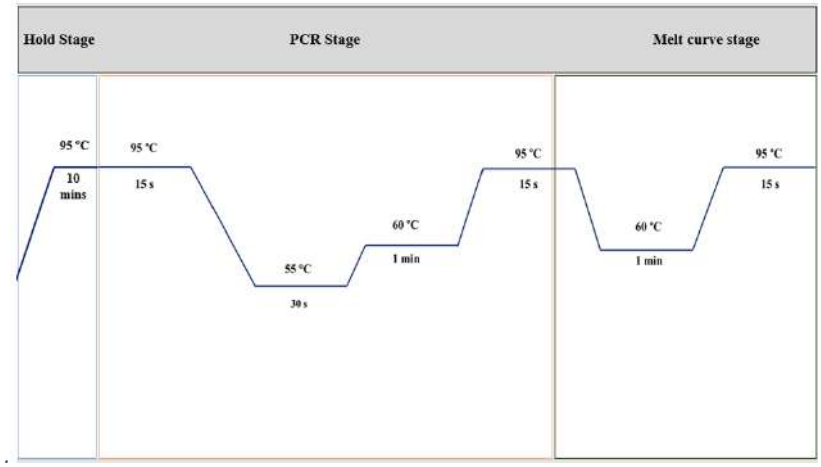

Figure 15. Reaction Condition for qPCR

2.4.Results:

2.4.1.Western Blot Ananlysis Confirms High Purity of Synaptoneurosome Preparation

Western blotting was performed to assess the purity of the synaptoneurosome fraction. SynapsinI, a presynaptic marker, and PSD-95 (Post-Synaptic Density Protein 95), a postsynaptic marker, were both enriched in the synaptoneurosome fraction, confirming the presence of synaptic components. In contrast, Glial Fibrillary Acidic Protein (GFAP), a glial marker, was predominantly detected in the total lysate, indicating minimal glial contamination in the synaptoneurosome preparation. βIII-tubulin (Tuj1) was used as a loading control.

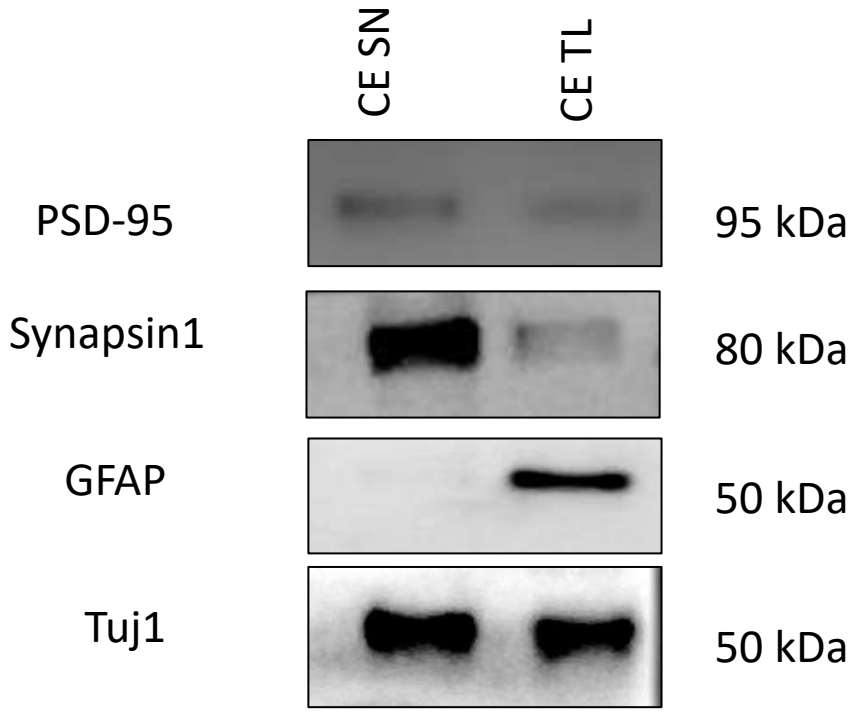

Figure 16. Western blot images for the validation of synaptoneurosome.

2.4.2. qPCR analysis reveals a significant reduction in Gas5 lncRNA reduction in Heterozygous Neurexin1 mutant male rats.

lncRNAs have emerged as key regulatory molecules in synaptic function and neurodevelopment, both of which are profoundly affected in autism spectrum disorder (ASD). GAS5 has been implicated in neuronal gene regulation and shows co-expression with other ASD-associated lncRNAs (Taheri et al., 2021). Although not differentially expressed between ASD and control groups in plasma, GAS5 forms part of a highly correlated lncRNA network, suggesting its indirect regulatory influence in ASD pathophysiology. Another lncRNA PVT1, is enriched in synaptic compartments (Samaddar et al., 2023). Moreover, PVT1 has been recently shown to be downregulated in the peripheral blood of kids with ASD (Jiang & Chen, 2023). To assess their

expression levels in the cerebellar synapse of Neurexin1 KO ASD rat model, qPCR analysis was done.

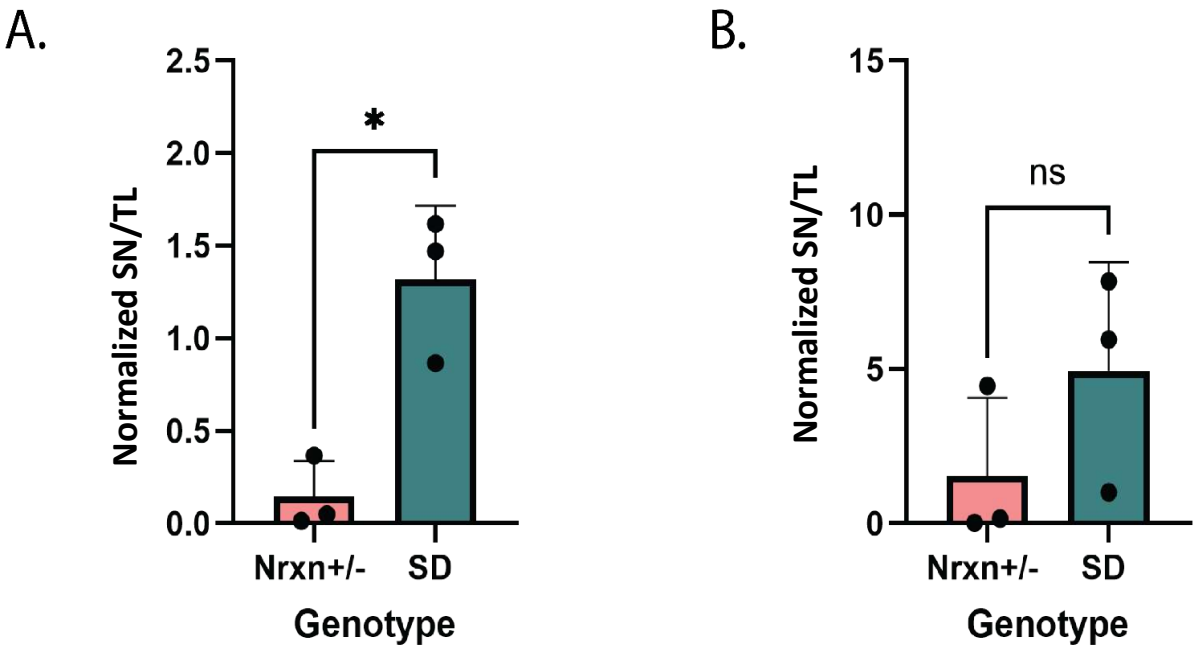

Figure 17. Quantification of lncRNA by qPCR.Significantly decreased expression of Gas5 (p=0.02) was observed in heterozygous Neurexin1 mutant rats. B. Expression of Pvt1 between the two genotypes. (SD= Sprague Dawley/Control rats; Nrxn+/-= Heterozygous Neurexin1 KO rats). All data are expressed as means ± SEM. A two-tailed t-test was performed.

RNA was extracted from synaptoneurosomes and total lysates to examine the variations in long non-coding RNA (lncRNA) expression in synaptic compartments. This RNA was subsequently reverse-transcribed into cDNA and subjected to quantitative PCR (qPCR). The ΔCT and ΔΔCT methods were used to normalize the levels of lncRNA with the control marker Tuj1.

Normalised SN/TL expression of Gas5 was found to be significantly lower in the heterozygous Nrxn1 mutant male rats *(p= 0.02)*. No significant change was seen in the case of Pvt1 expression in both groups (Figure 17.).

2.5.Discussion:

2.5.1. qPCR analysis reveals a significant reduction in Gas5 lncRNA reduction in Heterozygous Neurexin1 mutant male rats

qPCR analysis results revealed a significant reduction in expression levels of GAS5 in the heterozygous neurexin1 KO model (p=0.02). GAS5 regulates miR-21 and PTEN, hence influencing the AKT pathway. The AKT pathway is involved in cell survival, growth, and metabolism. Increased GAS5 expression has been reported to inhibit the AKT pathway, while decreased levels of GAS5 can activate the AKT pathway. GAS5 can bind to miR-21, acting as a sponge and increasing PTEN levels, which in turn inhibits the AKT pathway (Zhou & Chen, 2020). Further studies are warranted to directly assess miR-21 and PTEN expression levels, as well as the phosphorylation status of key AKT pathway components, in the *neurexin1* KO model to experimentally validate this proposed regulatory axis. Investigating the functional consequences of AKT pathway activation in these models would also provide crucial insights into the precise role of *GAS5* in *neurexin1*-related cellular processes.

In addition to its role in signalling, GAS5 variants also interact with proteins such as CAPRIN1 and G3BP2, that are involved in RNA translation and stress granule assembly at the synapse. By influencing these proteins, GAS5 can possibly impact protein synthesis at the synapse.

No significant change in PVT1 expression was seen between the two genotypes, suggesting that its expression may not be altered in this model or may be regulated under different consitions or developmental stages.

Chapter 3: Gait Alterations in ASD Rat Model: Neurexin1 Knock Out

3.1. Introduction

3.1.1. Locomotion

The stance phase in humans is characterized by heel touch which is distinct from what is seen in rodents, where the entire paw almost touches the ground simultaneously with most of the pressure exerted by the metatarsal and metacarpal pads on the hind and fore legs, respectively. The pressure is gradually transferred rostrally to the toes before liftoff during the stance phase (Mendes et al., 2015).

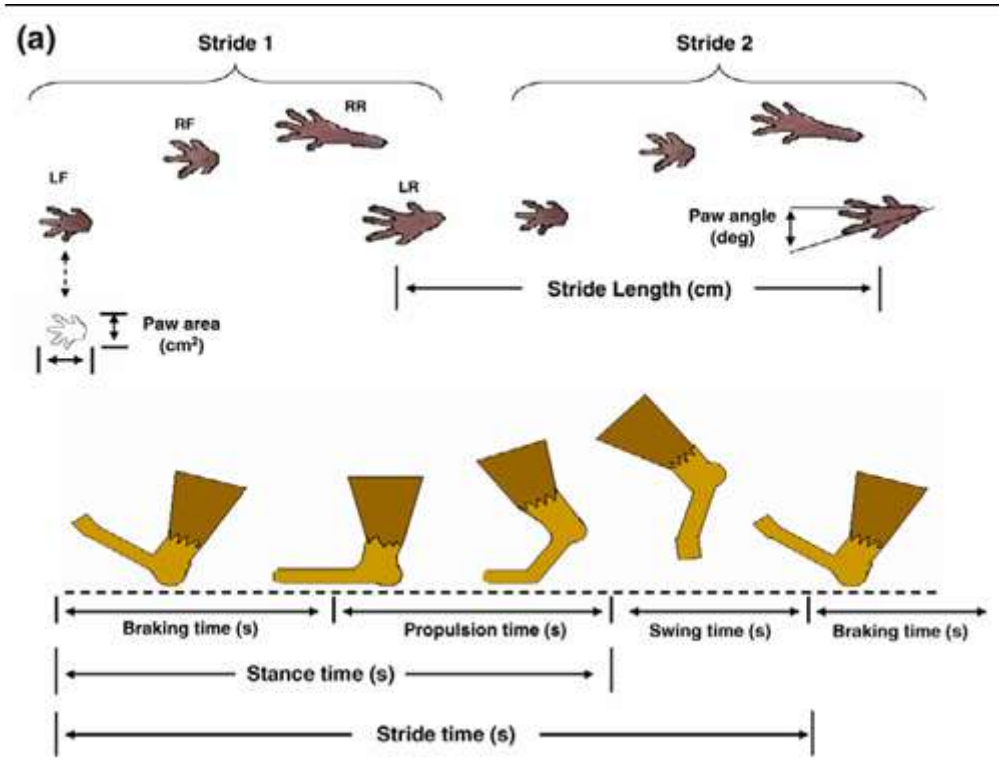

Figure 18. Illustration demonstrating swing and stride from bottom view (upper) and lateral view (lower) (Vincelette et al., 2007).

Individuals with autism have been documented to have an abnormal gait. Reduced stride length, increased cadence, increased variation in stride width, and reduced range of motion among other issues (Kindregan et al., 2015). Deficits in motor development as a symptom of autism is relatively a recent school of thought. Early views underscored that motor development in ASD-afflicted kids is similar to their neurotypical counterparts (Hallett et al., 1993; Mayes & Calhoun, 2003) or perhaps even more advanced (Johnson et al., 2007; Rimland, 1964).

Several motor deficits associated with ASD, like vestibular control and fine and gross motor abnormalities, have also been seen in patients with known cerebellar defects (Takarae, Minshew, Luna, & Sweeney, 2004). Abnormal saccadic eye movements and deficits in smooth pursuit are suggestive of vertical dysfunction in ASD (Takarae, Minshew, Luna, Krisky, et al., 2004). fMRI studies have shown reduced ipsilateral activation of the cerebellum during gross motor movement and diffuse activation in lobules VI-VII (*Differential Effects of Developmental Cerebellar Abnormality on Cognitive and Motor Functions in the Cerebellum: An fMRI Study of Autism | American Journal of Psychiatry*, n.d.; Mostofsky et al., 2009). Furthermore, reduced Purkinje and granule cells (Fatemi et al., 2012), and vermal hypo- and hyperplasia have also been reported. Pierce and Courchesne have also reported a reduced number of lobules in the cerebellar vermis correlating with decreased exploratory activity in Autistic kids (Courchesne et al., 2001)

3.2. Aims and Objectives:

To evaluate motor behavior in Neurexin1 (NRXN1)) knockout models and correlate behavioral changes with molecular alterations.

a) Design and standardize behavioural set up to analyse rodent gait.
b) Acquire videos to analyse gait.
c) Develop DeepLabCut model to annotate videos.
d) Extract gait parameters from the annotated videos.
e) Linear Mixed Effect Modelling to compare gait differences in Neurexin1 mutants and wild type rats.

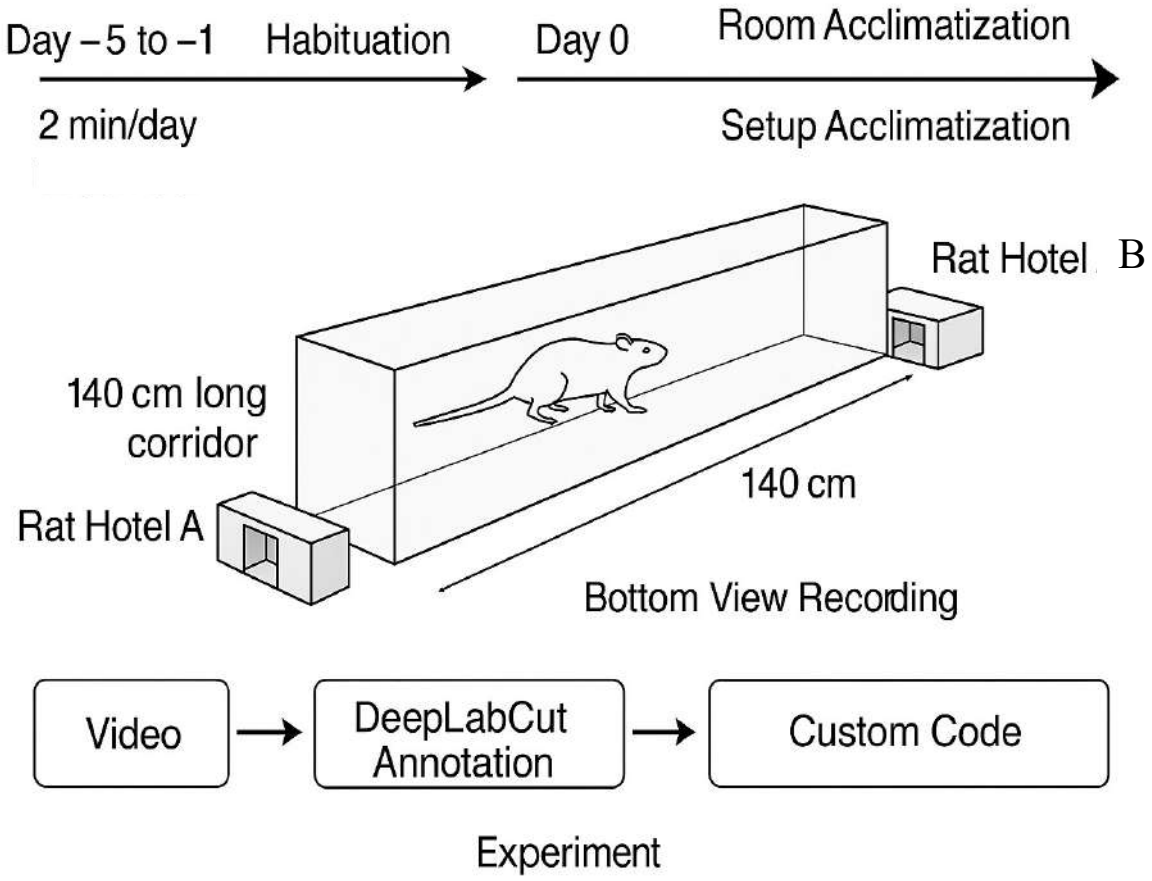

Figure 19. Locomotion and gait analysis paradigm

3.3. Materials and Methods

Gait Analysis and Locomotor behaviour

Apparatus:

- Rat Hotels: There are two homecages (40cm x 25cm x 20cm) made out of acrylic sheets on both ends of the corridor. Food and water are provided ad libitum on both ends.
- Corridor: A transparent corridor of length 140cm (width= 9cm and height= 30cm) with adjustable width.

  The entire structure is supported by a metallic frame.
- Camera: BFS-U3-16S2C-CS USB 3.1 Blackfly® S, Color Camera is mounted to the metallic rack to capture the bottom view video of rats passing.

Animals: P70- P90 Sprague Dawley and Neurexin1 KO rats with Sprague Dawley background were issued and habituated for 5 days for 2 minutes each day. The rats were allowed to acclimatize to the room 30 minutes before the video acquisition and were familiarized with the apparatus for 10 minutes.

Video Acquisition: The rats are acclimatized to the room 30 minutes before before video acquisition. The weight of the rats is taken before any video acquisition. They are gently put into the apparatus from one end. After 10 minutes, videos of the rats traversing the corridor are taken at 120fps. The rats were given no incentive or reward to walk, their native locomotion is recorded. Videos in which rats do not traverse the entire corridor are still analysed, focusing exclusively on the segments where the animals are running or walking. To maintain consistency and simplify analysis, only left-to-right crossings were included and a buffer length of 20cm on both ends was kept. 10-20 Passages were collected in each of 3 daily sessions.

Video Annotation: The videos were annotated using a machine learning algorithm called DeepLabCut. The labelling was done as follows:

Frontright1, Frontleft1, Hindright1, Hindleft1: Junction of the two middle fingers

Frontright2, Frontleft2, Hindright1, Hindleft1: Imaginary junction where the first and last finger would meet

Midleft: Half the distance between frontleft midpoint and hindleft midpoint; similarly for Midright.

Bodymid: Half the distance between nose and tailbase.

Figure 20. Illustration for DeepLabCut annotation.

DeepLabCut Model: DeepLabCut (Mathis et al., 2018) allows for markerless pose estimation which allowed me to observe the animal in its natural state without disturbing it by handling too much. The DeepLabCut model was trained using video recordings obtained from five postnatal day 70 (P70) rats. A total of approximately 500 video frames were manually annotated and subsequently divided into training and testing datasets, with 80% of the frames allocated for training and 20% for testing. The model was trained over 500 epochs using the ResNet-50 backbone architecture. The training was optimized to achieve a test root mean square error (RMSE) of less than 5 pixels, indicating high precision in key-point detection.

As seen in Figure 21., diagonal limbs are usually in the same phase and anti-phase is maintained between the left and right paws. Overall, in normal gait patterns, both temporal and spatial symmetry is maintained.

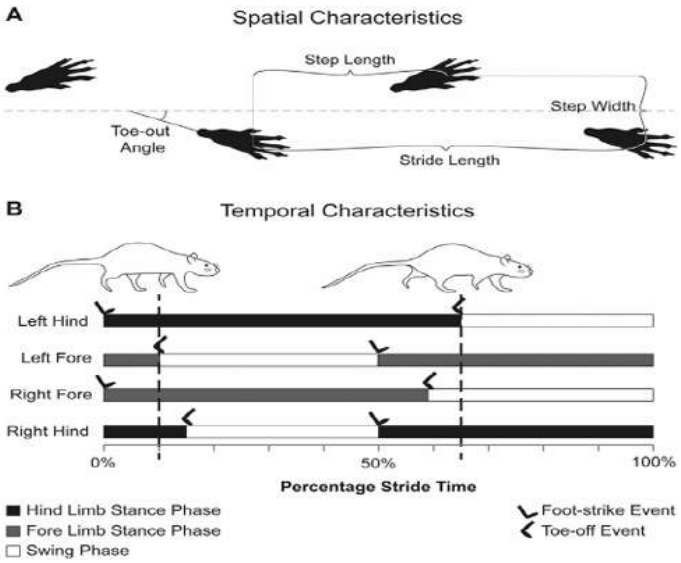

Figure 21. Spatial and Temporal Gait Characteristics (Lakes & Allen, 2016).

Limb Parameters	Definition
Stride Length	The displacement of a single paw between two consecutive heel strikes.
Stance Duration	Time in milliseconds that foot is on the ground during stride.
Swing Duration	Time in milliseconds that foot is in the air during stride.
Stride Frequency	Number of strides completed in one second.
Stance phase	Relative timing of limb touchdowns to stride cycle of reference paw. Calculated as: (stance time−stance time reference paw)/stride duration.
Limb Duty Factor	The portion of time when a limb is in contact with the ground during a stride cycle. (Stance duration/Stride duration)
Stance Width	The distance between the front paws or the hind paws when both are in the stance phase.

Table 10. Gait parameter definitions

Any coordinates below 0.8 were discarded and missing data points were interpolated. Savitzky Golay Filter with a window size of 7 and 3 polynomial was applied to remove jitter in the data. Further gait parameter extraction was done using custom code on Google Colab.

Strides were categorized based on the relative position and displacement of the paw (Fiker et al., 2020). Frames in which the paw displacement significantly exceeded the average displacement were classified as swing phases, indicating paw movement. In contrast, frames, where the paw remained relatively stationary, were classified as stance phases, corresponding to periods of ground contact and support. One complete cycle of a paw, consisting of a stance phase transitioning into a complete swing phase was classified as a stride.

Stride duration, stride length, limb duty factor, stance duration, swing duration, bodymid speed, tailend speed, stance width, and interlimb coordination were extracted.

Statistics: Considering the nested nature of data with varying numbers of trials per animal, data analysis and statistical analysis were performed with linear mixed models

All statistical analyses for the behaviour paradigm were conducted using Python and R. For each comparison, linear mixed-effects models were constructed and evaluated by comparing models with additive fixed effects to those including interaction terms, using likelihood-ratio tests, and assessing the statistical significance of model terms. Initially, **genotype, paw, speed, and body weight** were considered as fixed effects. However, due to the low variability of body weight (n=6 subjects), this variable was subsequently excluded from the final model. Similarly, an interaction term between **paw and genotype** was initially considered to investigate whether the effect of genotype differed across individual paws. However, due to the limited sample size (n=6) and concerns regarding statistical power, this interaction term was also excluded from the final model. No collinearity was found between the fixed effects.

Term	VIF	Tolerance	Conclusion
Genotype	1.00	1.00	No collinearity
Paw	1.00	1.00	No collinearity
Speed_m.s	1.00	1.00	No collinearity

Table 11. Variance Inflation Factor (VIF) values assessing multicollinearity among fixed-effect predictors in the linear mixed-effects model.

All models included **random intercepts for individual animals** to account for subject-level variability.

Model assumptions of linearity, homoscedasticity, and normality of residuals were assessed through diagnostic plots (residual vs. fitted, Q-Q plots, etc.). No major violations were observed.

3.4. Results

3.4.1. Gait parameter extraction

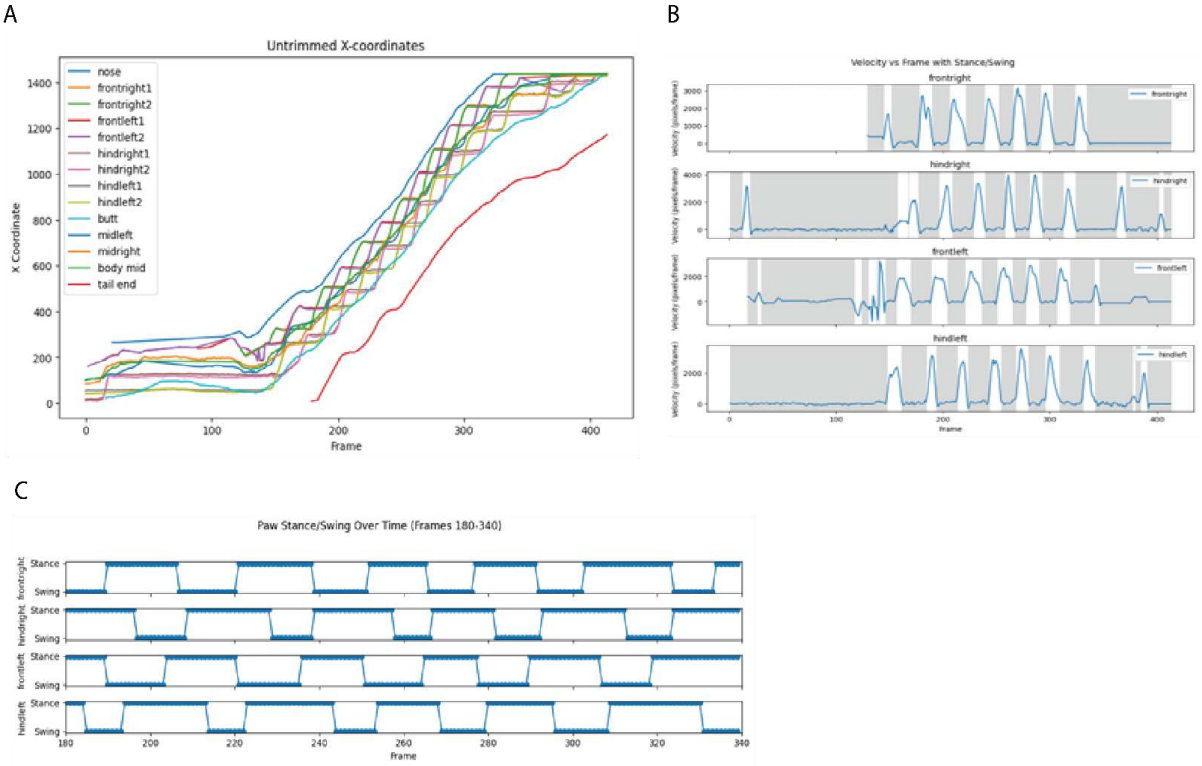

Figure 22. Pre-processing for gait parameter extraction. The X-coordinates for each labelled body part are plotted (A), swing and stance are categorized on the basis of differential paw position, this is confirmed by velocity peaking mid-swing as shown in (B). Time frames of clear walking behaviour are isolated and further analysed (C).

The segments of the video to be analysed were extracted as per Fig 22. After extraction of the parameters, the normality of the data was checked using the Shapiro-Wilk test. On confirmation of normality, further tests were applied.

A significant reduction in body weight was observed in heterozygous *Neurexin1* knockout rats compared to controls ($p = 0.0055$, two-tailed t-test). Gait parameters such as body mid-speed, stride duration, stride length, stride frequency, and duty factor did not show any

statistically significant differences between groups; however, a consistent trend was observed (Fig 15.).

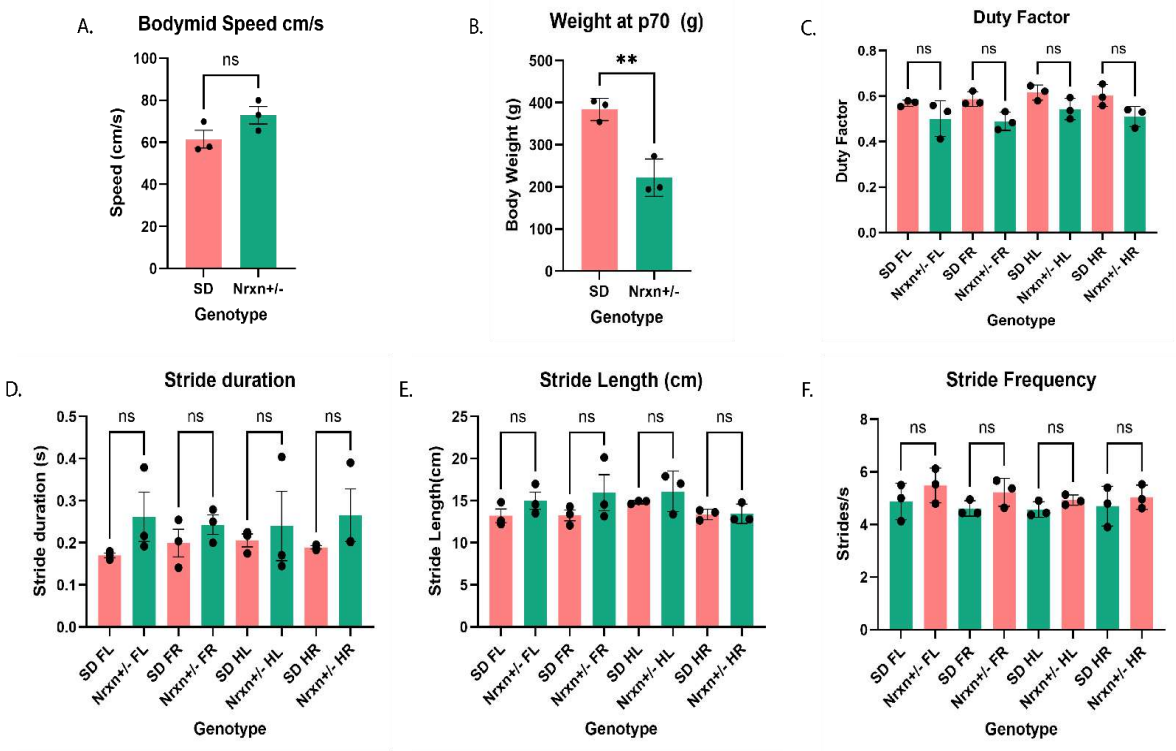

***Fig 20. Gait Analysis**: A to F show comparisons of Bodymid Speed (cm/s), Weight at p70 (g), Duty Factor, Stride Duration (s), Stride Length (cm), Stride Frequency in Sprague Dawley (SD) vs Heterozygous Neurexin1 KO (Nrxn+/-) p70 males rats (FL: Front Left, FR: Front Right, HL: Hind Left, HR: Hind Right). All data are expressed as means ± SEM. One way ANOVA followed by Tukey's multiple analysis was performed. (*p < 0.05, **p < 0.01, ***p < 0.001).*

3.4.2. Linear Mixed Effect Modelling to evaluate gait parameters

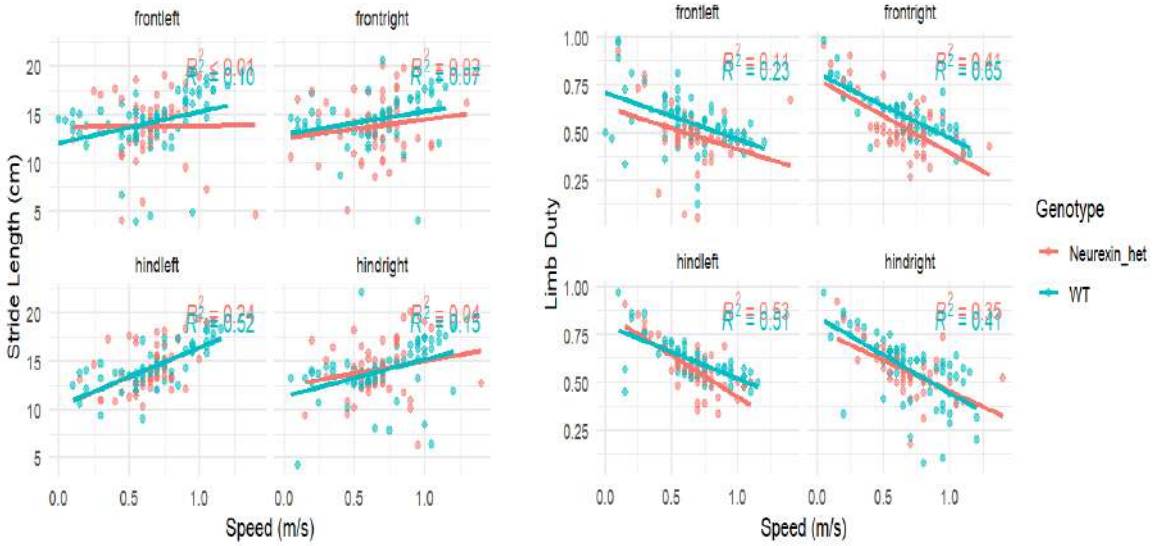

Figure 23. Relationship between speed and stride length (left panel) or limb duty factor (right panel) across different paws.

Linear regressions were performed separately for each genotype (Neurexin_het in red and WT in blue) within each paw (frontleft, frontright, hindleft, hindright). Each point in Fig 23. represents an individual stride. Shaded lines depict linear trends, and the coefficient of determination (R^2) is displayed for each fit. These plots were used to assess linearity assumptions and explore genotype-specific trends in gait parameters.

	Y					
Y~ fixed effects + re	**Stride length (cm)**		**Stride duration (s)**		**Limb Duty**	
	R^2m	R^2c	R^2m	R^2c	R^2m	R^2c
*Y~Genotype*Paw + re*	0.0071	0.1403	0.0025	0.1305	0.035	0.2409
*Y~Genotype*Paw+ Speed + re*	0.1043	0.2452	0.2150	0.2150	0.3803	0.3875
*Y~ Genotype*Paw+ Speed+ weight + re*	0.1056	0.2734	0.2120	0.2137	0.3721	0.3866
*Y~Genotype*Paw+ Speed *weight+ re*	0.1065	0.2675	0.2016	0.2211	0.3739	0.3863

re= random effects= 1| Individual

R^2m: marginal; R^2c: conditional was obtained from generalized linear mixed effects model

Table 12. Linear Mixed Model Equations to explore differential effect of genotype on different paws

A likelihood ratio test comparing models with and without the *Speed* × *Body Weight* interaction revealed no significant improvement in model fit for any of the gait parameters examined. For Stride Length, the interaction term did not significantly enhance the model ($\chi^2(1) = 0.32$, $p = 0.57$), and the simpler model—where speed remained a significant predictor—was retained. Similarly, for Stride Duration, the interaction was not significant ($\chi^2(1) = 1.60$, $p = 0.21$), favoring the simpler model. For the Limb Duty Factor, the interaction again did not improve model fit ($\chi^2(1) = 0.61$, $p = 0.44$), further supporting the use of the reduced model.

The interaction between genotype and paw was initially included to test whether the effect of genotype varied across different paws. However, ANOVA model comparisons revealed that models without the interaction term provided a better fit (lower p-values; p-value< 2.2e-16) for all dependent variables considered.

Model	Fixed Effects	Limb Duty			Stride Length		
		p-value	R^2m	R^2c	*p*-value	R^2m	R^2c
m1	Genotype	–	0.004	0.208	–	0.000006	0.134
m2	Genotype + Paw	0.00063 ***	0.031	0.238	0.512	0.004	0.138
m3	Genotype + Paw + Speed	**< 2.2e-16 *** **	**0.378**	**0.385**	**7.6×10^{-12}**	**0.102**	**0.243**
m4	Genotype × Paw + Speed	0.340	0.3803705	0.3875086	0.546	0.104	0.245

* All models have the random effect (re); re= random effects= 1| Individual

R^2m: marginal; R^2c: conditional was obtained from generalized linear mixed effects model

Table 13. Simpler Linear Mixed Effects Model more suitable for the current dataset.

Model 'm3' (Table.) was found to be the best fit among all models. Speed was found to have a significant impact on the gait parameters. Marginal R^2 explains the proportion of variance explained only by the fixed variables and Conditional R^2 explains the variance explained by both fixed and random variables. 37.8% of the variance in limb duty is explained by fixed variables in the model m3 and 38.5% of the variance is explained by the combination of both fixed and random variables. Similarly, 10.2% of the variance in stride length is explained by fixed variables and 24.3% variance is explained by the combination of both fixed and random variables.

Dependent Variable	Equation
Limb Duty	0.708+0.041·GenotypeWT+0.040·PawFront Right +0.075·PawHind Left+ 0.050·PawHind Right−0.317·Speed (m/s)
Stride Length	11.908-0.168·GenotypeWT+0.20·PawFront Right +0.386·PawHind Left-0.3869·PawHind Right+3.5821·Speed (m/s)

Table 14. Equations depicting contribution of fixed effects on Limb Duty and Stride Length.

Pairwise comparisons of estimated marginal means (EMMs) for stride length and limb duty were conducted between genotypes within each paw using the emmeans package with Tukey adjustment for multiple comparisons. No significant differences were found.

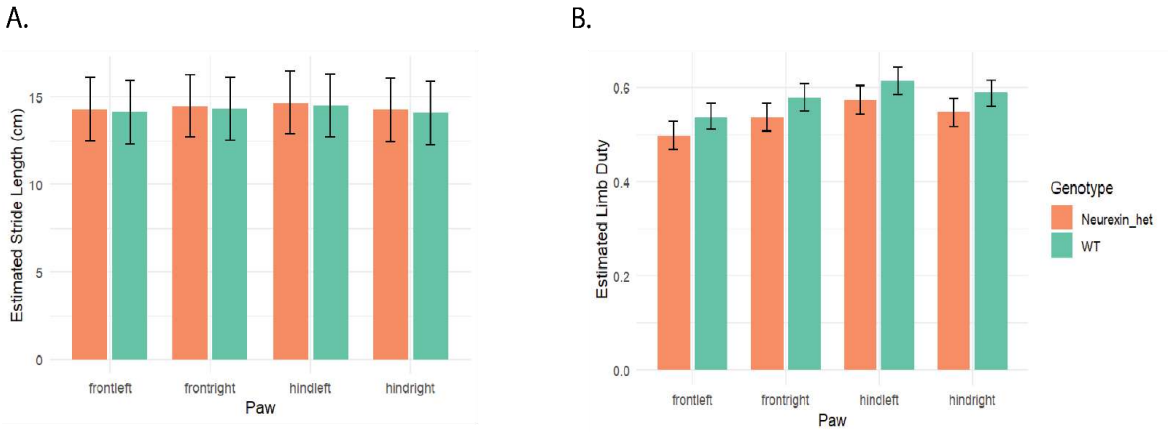

Figure 24. Pairwise comparisons of estimated marginal means (EMMs) for stride length and limb duty factor were conducted between genotypes within each paw. Error bars represent 95% confidence intervals. The differences were not found to significant.

Although not significant, Neurexin heterozygous rats exhibited a reduced limb duty compared to wild-type animals (estimated difference = −0.0408, SE = 0.0143, $p = 0.054$), independent of paw. This uniform reduction suggests a global alteration in stance phase duration. There is also a slight increase of 1.56cm in stride length of heterozygous Neurexin rats (estimated difference= 1.56, SE=2.96, $p= 0.6355$).

3.5. Discussion

Motor deficits such as impaired coordination, abnormal motor symmetry, and hyperactivity have been documented in individuals with ASD (Yirmiya & Charman, 2010), yet they remain excluded from current diagnostic criteria. While gait abnormalities have been characterized in mouse models (Khairullin et al., 2024; Matas et al., 2021) often showing marked alterations across multiple gait parameters, these findings are frequently derived from paradigms like the rotarod or involve repeated experimenter handling. Few studies investigate spontaneous, unforced locomotion in a naturalistic setting, which may offer more ecologically valid insights into motor phenotypes associated with ASD. In this thesis, I have tried to study the native locomotion of rats, allowing subjects to walk without any external motivation.

A significant change in bodyweight was seen between the genotypes. Since, gait is a function of body weight (Machado et al., 2015), one would expect alterations in gait parameters across genotypes. Surprisingly, there weren't any significant changes across the genotypes.

To investigate the contributions of various factors to gait, linear mixed-effects model (LMMs) was employed. Speed significantly influenced stride length, limb duty factor, and stride duration, highlighting its critical role in gait modulation**.** This observation aligns with findings from previous studies, which consistently report that locomotor speed affects various gait parameters, likely due to underlying biomechanical and neuromuscular adjustments required at different speeds (Machado et al., 2015; Pitzer et al., 2021). However, the current models explained only a modest proportion of the variance in gait parameters (e.g., marginal $R^2 \sim 0.1$), and none of the fixed effects reached statistical significance in several of the models. This limited explanatory power likely reflects the small sample size (n = 6 animals), which constrains the ability to detect subtle genotype effects or interactions reliably.

Pairwise comparisons were made for every genotype on every single paw using the linear mixed-effects model. To account for multiple comparisons and identify significant differences between genotypes, these comparisons employed the least squares mean (LSM) method. Neurexin1 KO heterozygous rats showed a significant trend of decreased estimated limb duty ($p = 0.054$). This finding suggests that these animals may have a different gait characteristic, particularly with regard to the percentage of time the limb spends in ground contact, even though it does not reach conventional statistical

significance. Further evidence that any possible motor differences may be found in the temporal aspects of gait rather than spatial measures comes from the lack of a discernible change in stride length. Because of the comparatively small sample size and the borderline p-value. These findings should be interpreted with caution. Future studies with increased power and potentially higher-resolution kinematic analyses are needed to confirm this preliminary observation and elucidate the precise nature of this potential motor deficit in Neurexin heterozygous models.

Despite the insights gained from this study, several limitations warrant consideration. Notably, anxiety related behaviour is common in heterozygous Neurexin1 knockout rats (Achterberg et al., 2025; Wu et al., 2023), could potentially confound the interpretation of gait parameters. Furthermore, the assessment of locomotion was conducted under standard laboratory lighting condition. Ideally, to better capture the natural behaviour of rodents, which are nocturnal creatures, locomotion should be assessed at night in the dark phase using IR illumination and IR sensitive cameras.

Finally, while 2D analysis provided some insight, use of 3D pose estimation could offer more robust and comprehensive analysis of gait. By incorporating techniques such as setting up a mirror beneath the apparatus at a 45-degree angle, simultaneous top-down and ventral views can be captured, enabling the reconstruction of limb movements in three dimensions. This 3D approach would provide empirical proof of specific gait phases, such as swing and stance, through observed changes in the Z-axis (vertical movement), offering a richer understanding of kinematic details that are not fully accessible from a 2D perspective.

Future work will focus on increasing the sample size to improve the power and robustness of statistical inference. Larger datasets will allow for better estimation of fixed effects, exploration of potential interactions, and potentially inclusion of more complex random effects structures to capture individual variability more accurately.

Together, these findings suggest that heterozygous Neurexin1 KO rats exhibit both molecular and behavioural alterations that may be mechanistically linked. Decrease in GAS5 expression observed in the qPCR analysis raises the possibility that reduced level of GAS5 may impair synaptic protein synthesis. Such disruptions may, in turn, affect motor circuit maturation or plasticity. Although overt gait differences across genotypes were not statistically significant, the observed trend toward decreased limb duty aligns with emerging evidence that lncRNA such as GAS5, can influence neuronal function and

behaviour. These preliminary data warrant further investigation to fortify molecular to behavioural axis in Neurexin1 mutant models.

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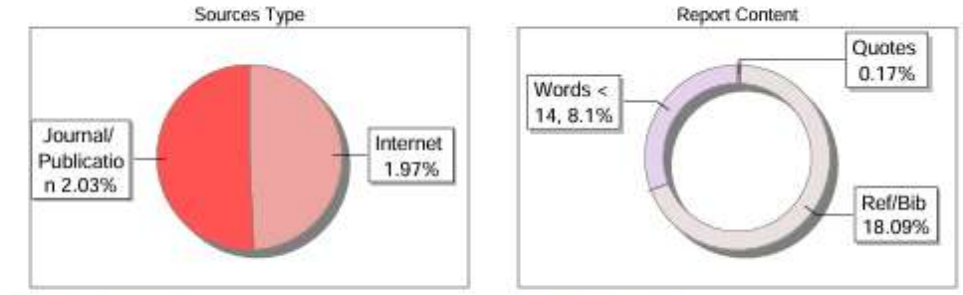

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